Isolation and Characterisation of Antimicrobial Compounds from *Antizoma angustifolia*

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(B.Pharm.)

Dissertation submitted in the partial fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

in the

School of Pharmacy (Pharmaceutical Chemistry) of the Faculty of Health of Sciences

at the

North-West University (Potchefstroom campus)

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Potchefstroom

2004

"Education is the great engine to personal development. It is through education that the daughter of a peasant can become a doctor, that the son of the miner can become the head of mine, that the child of a farmer can become the president of the great nation. It is what we make of what we have, that separates one person from another"

Nelson Mandela

ABSTRACT

Infectious diseases are responsible for more than 17 million deaths per year worldwide, most of which are associated with bacterial infections. The increase in antibiotic resistance is thought to be a contributing factor to this problem. It is thus clear that more antimicrobials with different mechanisms of action are needed to help alleviate the problem. Isolation of antimicrobial compounds from plants could contribute towards solving this problem as they may have different mechanisms of action than the antimicrobial agents currently in use.

The aim of the study was to identify a specific plant with antimicrobial activity and to isolate and characterise the compounds responsible for this activity.

Eight plants, namely *Antizoma angustifolia, Carpobrotus acinaciformis, Delosperma herbeum, Melianthus comosus, Physalis viscosa, Rhus pyroides, Zanthoxylum capensis and Ziziphus mucronata* were selected for screening. Soxhlet extraction was used to prepare extracts of the different morphological parts of each plant using petroleum ether, dichloromethane, ethyl acetate and ethanol successively. These plant extracts were screened for antimicrobial activity against a range of microorganisms using disc diffusion and microplate methods. The extracts showed variable activity with the dichloromethane extract of *Antizoma angustifolia* leaves showing the most promising activity.

The leaf extracts (dichloromethane, ethyl acetate and ethanol) of *Antizoma angustifolia* were subjected to activity-guided fractionation using column chromatography. This lead to the isolation of bulbocapnine and dicentrine from the dichloromethane extract and the isolation of crotsparine from the dichloromethane, ethyl acetate and ethanol extracts. The compounds were identified by spectroscopic techniques. These compounds were evaluated for antimicrobial activity using the microplate method and crotsparine showed weak activity.

Although the activity of crotsparine was not very high, it might still be useful as a lead compound in the development of antimicrobial drug development. The biological activity of these compounds does however confirm the fact that the diverse chemistry of plants is still a very important source of novel biologically active and lead compounds. The biological activity of the compounds isolated from *Antizoma angustifolia* could justify its ethnopharmacological uses.

OPSOMMING

Infeksies veroorsaak jaarliks wêreldwyd meer as 17 miljoen sterftes en die meeste van hierdie infeksies word deur bakterieë veroorsaak. Die toename in weerstandigheid teen antibiotika vererger hierdie probleem. Nuwe antimikrobiese middels wat volgens ander meganismes werk kan help om hierdie probleem te oorkom. Die isolasie van antimikrobiese verbindings uit plante kan dus 'n bydra tot die oplossing van hierdie probleem lewer deurdat hierdie verbindings dalk volgens 'n ander meganisme as die huidige antimikrobiese middels mag werk.

Die doel van hierdie studie was om 'n spesifieke plant met antimikrobiese aktiwiteit te identifiseer en om die aktiewe verbindings te isoleer en te karakteriseer.

Agt plante, naamlik *Antizoma angustifolia, Carpobrotus acinaciformis, Delosperm herbeum, Melianthus comosus, Physalis viscosa, Rhus pyroides, Zanthoxylum capensis* en *Ziziphus mucronata,* is vir sifting gekies. Soxhletekstraksie met opeenvolgend petroleumeter, dichloormetaan, etielasetaat en etanol is gebruik om ekstrakte van die verskillende morfologiese dele van elke plant te verkry. Hierdie plantekstrakte is met die plaatdifussie- en mikroplaatmetodes vir antimikrobiese aktiwiteit teen 'n reeks mikro-organismes getoets. Die ekstrakte het wisselende aktiwiteit vertoon met die mees belowende aktiwiteit in die dichloormetaanekstrak van die blare van *Antizoma angustifolia.*

Die blaarekstrakte (dichloormetaan, etielasetaat en etanol) van *Antizoma angustifolia* is met behulp van kolomchromatografie gefraksioneer terwyl die biologiese aktiwiteit as riglyn gebruik is. Dit het bulbokapnien en disentrien uit die dichloormetaanekstrak gelewer en krotsparien uit die dichloormetaan-, etielasetaat- en etanolekstrakte. Die verbindings is met behulp van spektroskopiese tegnieke geïdentifiseer. Hierdie verbindings is met die mikroplaatmetode vir antimikrobiese aktiwiteit ondersoek waartydens krotsparien swak aktiwiteit vertoon het.

Hoewel die aktiwiteit van krotsparien nie baie sterk is nie, kan dit steeds nuttig wees as leidraadverbinding vir die ontwikkeling van antimikrobiese geneesmiddels. Die biologiese aktiwiteit van hierdie verbindings bevestig egter die feit dat die rykdom in die chemie van plante steeds 'n baie belangrike bron van nuwe biologies aktiewe en leidraadverbindings is. Die aktiwiteit van die verbindings uit *Antizoma angustifolia* kan ook die etnofarmakologiese gebruik daarvan regverdig.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to almighty God for granting me the opportunity, ability, strength and courage to complete this dissertation.

To my parents and siblings, thank you for your love, support, patience and faith in me. I dedicate this dissertation to you all.

To Prof. S.F. Malan (supervisor), thank you for your help, guidance support and your valuable time.

To Prof. J.C. Breytenbach (assistant supervisor), thank you for your expert advice, guidance and time throughout the study. It has been great working with you.

To Dr. S. van Dyk (co-supervisor), thank you for your valuable assistance and advice.

To all Pharmaceutical Chemistry personnel, thanks for your co-orporation.

To colleagues and friends, in particular, Reuben, Kenny, Lebogang, Gorden, Lerato, Mishack, Susan, Mzwai thanks for your friendship, love, assistance and encouragement.

To my partner, Lebogang and my daughter Rorisang, thanks for your constant love and support.

Thanks to Canon Collins for financial support.

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Chapter 1: Introduction

1.1 Introduction

Infectious diseases are responsible for more than 17 million deaths per year worldwide, most of which are associated with bacterial infections (Hagan *et al.*, 2002; Picard & Bergeron, 2002). This might be due to among other things, lack of resources to combat these diseases, especially in the developing countries. However, developed countries like the USA are also affected by this problem. It is estimated that infectious diseases are the underlying cause of death in 8% of the deaths occurring in the USA (Pinner *et al.*, 1996). These alarming statistics are believed to be attributed to an increase in respiratory tract infections and HIV/AIDS. The increase in antibiotic resistance is also thought to be a contributing factor to this problem. When antibacterial drugs were introduced into clinics in the 1930s, it was believed that infectious diseases would be controlled and eventually mastered. Although antibiotics have saved many lives and have transformed the medical profession, the above-mentioned statistics are proving that belief wrong, as infections remains the leading cause of death worldwide (Williams, 2002). Looking at these statistics, it is clear that more antimicrobials with different mechanisms of action are needed to help alleviate the problem.

Since the advent of antibiotics, the use of plant derivatives as antimicrobials has been virtually nonexistent, because pharmaceutical companies were relying on fungi and bacteria as sources of antimicrobials (Cowan, 1999). However, some plant products are also known to have antimicrobial activity even against microorganisms that are known to be resistant to some of the drugs currently used. For example, artemisinin (formally called arteannuim and also called qinghaosu in China) isolated from *Artemisia annua* is reported to have antimalarial activity against among others, chloroquine resistant and piperaquine-resistant malaria parasites (Klayman, 1985). Plants could provide a solution to the problem of drug resistance, as they may act by different mechanisms than the presently used antibiotics (Eloff, 1998a). On the other hand, the World Health Organisation (WHO) estimates that about 80% of the people in the developing countries use traditional or complementary/alternative medicine (TM/CAM) as part of primary health care. Traditional medicine is mainly based on the use of plant products as remedies. Therefore, medicinal plants used in traditional medicine should be studied for

safety, efficacy (Farnsworth, 1994) and potential to treat current drug resistant infectious diseases.

The aim of the study was to identify a specific plant with antimicrobial activity and to isolate and characterise the compounds responsible for this activity.

To reach the aim of this study the following objectives are proposed:

- To screen selected South African plants used in traditional medicine for antimicrobial activity.
- To isolate and characterise the compounds responsible for antimicrobial activity from the plant extract which show better antimicrobial activity among the selected plants.
- To evaluate the antimicrobial activity of the plant extracts and the isolated compounds.

Chapter 2: Plants and Medicines

2.1 Plants in the history of drug development

In antiquity, primitive people discovered that natural plants and certain animal parts had medicinal value. The big question is how they made those discoveries. There are quite a number of theories about this and amongst others are the following:

- Prehistoric humans learned by serendipity and then diffused knowledge throughout the world.

- Humans starving and forced to eat certain plants might have discovered their healing properties

- Humans acquired knowledge of medicinal plants by observing animals that seemed to use plants when they were sick.

One example of animal medicinal plant use is Lingusticum (lovage), a plant that is still used in the American and Mexican West. According to legend, the Navajo learned from bears that a *Lingusticum* species cured infestations, infections and stomach ailments. Alaska's kodiak bears dig up, chew, swallow, or rub their fur with roots and the brown bears at the Colorado springs zoo relish *Lingusticum* and also apply the chewed root to their faces and fur (Kay, 1993).

As a result of all these discoveries made, the study of material used for medicine was initiated and recorded as *Materia medica*. All the drugs that were utilised for their beneficial effects then came from natural living sources. For this reason, *Materia medica* or *Pharmacognosy*, was the most important pharmacological science (Albanese, 2003).

The period of using plants or animals or parts thereof for their beneficial health effects begun with primitive humans and lasted until the Middle Ages. During this era the treatment was based upon the use of the whole plant or some part of the plant such as leaves, bark, seeds, berries or fruits. Examples include belladona leaf or root (*Atropa belladona*), chinchona bark (*Cinchona pubescens*); digitalis leaf (*Digitalis lanata*); ma-

huang rhizome (*Ephedra sinica*); nux vomica seed (*Strychnos nux-vomica*) and poppy pod (*Papaver somniferum*).

With time, the use of plants and animals or parts thereof was abandoned for their more concentrated extracts, which greatly reduced therapeutic doses. Pharmaceutical dosage forms called galenicals were designed to extract and concentrate the active drug principles such as alkaloids, glycosides and volatile oils primarily from plants. Galenicals had an advantage over the direct use of plants, as it eliminated the need to use large quantities of plant material that had to be consumed or applied to achieve desired effects.

Further advancements were seen when galenicals were abandoned for their active ingredients (table 2.1). The German pharmacist, Sertuner was among the first people to isolate drugs from plants. He isolated the narcotic alkaloid morphine from extracts of the poppy plant (opium) as early as 1805. Other drugs, which were first isolated from plants, include strychnine (1818) from *Strychnosa nux-vomica*, quinine from chinchona bark and reserpine from *Rauwolfia serpentina* (Albanese, 2003).

Table 2.1: Periods in the history of drug development and examples (Albanese, 2003).

Use of plant parts	Poppy plant unripe pods
Use of extracts obtained from plants	Opium, extract from the poppy pod
Use of active ingredient isolated from plants	Morphine (the main drug present in opium) and codeine
Used of modified (semisynthetic) isolated active drugs from plants	Diacetylmorphine (heroin) a semisynthetic obtained from morphine, hydromorphone, oxymorphone, oxycodone
Pure chemical synthesis	Synthesis of meperidine, methadone, etc.

During the beginning of the twentieth century, isolated active drugs were modified (semisynthetics) to maximise therapeutic effects while minimising adverse effects.

In the 1950s, the use of pure synthetic chemicals started to expand and today it is the major source of drugs that are currently used for therapy. These chemicals are utilised

to treat diseases in different dosage forms such as tablets, powders, syrups, solutions etc. (Albanese, 2003).

2.2 The current use of traditional medicines

It is estimated by the WHO, that approximately 75-80% of the world's population uses plant medicines for their primary health care needs and about 85% of traditional medicine involves the use of plant extracts. For many this is from necessity, since they cannot afford the high cost of pharmaceutical drugs. A growing number of world health care consumers are turning to plant medicine for many reasons such as low cost and seeking alternatives with fewer side effects (Blythe, 1999). WHO (2003) defines traditional medicine as the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness. According to the Natural Products Alert (NAPRALERT) database, there are many records on medicinal plant use worldwide. The use of traditional medicines is common in countries like South Africa, China, India and many other countries in Africa, where medicinal herbs are sold in marketplaces alongside vegetables and other ware (Kong *et al.*, 2003).

Some countries such as China are incorporating traditional herbal medicine into modern health care systems. The blend of traditional herbal medicine, acupuncture, and Western medicine is China's unique answer to the health care needs of over one billion people. According to a survey conducted recently, almost 7300 plants have been used in traditional Chinese medicine. Chinese apothecaries contain a dazzling array of dried plant specimens, and prescriptions are filled, not with prepacked tablets or ointments, but with measured amounts of specific herbs. Economic factors also contribute to the reliance on indigenous cures, since the cost of manufactured pharmaceuticals is beyond the reach of most of the population.

In South Africa, traditional medicine plays an important role, where approximately 80% of the black population makes use of it (Jäger *et al.*, 1996). Unfortunately, all of the plant material (estimated to be 20000 tonnes per year) is harvested from the wild, which has led to overexploitation and severe threat to many medicinal plants (RCPGD, 2002). For instance, several plant species, such as wild ginger (*Siphonochilus aethiopicus*) and

the pepper-bark tree (*Warburgia salutaris*) have become extinct outside protected areas in Kwazulu-Natal in South Africa (Mander, 1998). It is therefore important that these plants are studied for their medicinal value before they become extinct.

The main problem facing the use of traditional medicines is the proof required to show that the active components contained in medicinal plants are useful, safe and effective. This is required to assure the medical field and the public regarding the use of medicinal plants as drug alternatives (Rukangira, 2003). In an attempt to respond to this problem, some scientists are testing plants extracts for biological properties and isolate, characterise and also test plant components for biological activities. Light *et al.* (2002) investigated the biological activities of one of the most important and threatened medicinal plants in South Africa, *Siphonochilus aethiopicus* (wild ginger). This plant showed significant antibacterial and anti-inflammatory activities. Viljoen *et al.* (2002a) also studied the chemical composition of the roots and rhizomes of the same plant (*Siphonochilus aethiopicus*). Although all plants parts (e.g. roots, leaves, flowers, bark etc) are used in South African traditional medicine, some parts are more commonly used than others. It was reported that nearly one third of the plant material used in South African traditional medicine is constituted of bark products (Grace *et al.*, 2002).

2.3 Contribution of plants to western medicine

Plant-derived medicines have made large contributions to human health and well-being. Today there are at least 120 distinct chemical substances derived from plants that are considered as important drugs currently in use in one or more countries in the world (Taylor, 2000). The study by Fabricant & Farnsworth (2001) showed that approximatly 80% of the plant-derived drugs they studied had an ethnomedical use identical or related to the current use of the active principle.

The goals of using plants as sources of therapeutic agents include:

- to isolate bioactive compounds for direct use as drugs, e.g., digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, vincristine;
- to produce bioactive compounds of novel or known structures as lead compounds for semisynthesis to produce patentable entities of higher activity and/or lower toxicity, e.g., metformin, nabilone, oxycodon (and other narcotic

analgesics), taxotere, teniposide, verapamil, and amiodarone, which are based, respectively, on galegine, Δ^9-tetrahydrocannabinol, morphine, taxol, podophyllotoxin, and khellin;

- to use agents as pharmacological tools, e.g., lysergic acid diethylamide, mescaline, yohimbine; and
- to use the whole plant or part of it as a herbal remedy, e.g., cranberry, echinacea, feverfew, garlic, ginkgo biloba, St. John's wort, Saw palmetto (Fabricant & Farnsworth, 2001).

It was reported that in industrialised countries, plants have contributed to more than 7,000 compounds produced by the pharmaceutical industry, including ingredients in heart drugs, laxatives, anticancer agents, hormone contraceptives, diuretics, antibiotics, decongestants, analgesic, anaesthetics, ulcer treatment and anti-parasitic compounds (WWF, 2003). At least 25% of prescription drugs in the USA contain at least one compound derived or originally derived from higher plants (Duke, 1990).

2.4 Synergy of phytochemicals

There is an experience based claim of phytotherapy that effects of plant extracts or constituents of herbal drugs are in many cases superior to isolated compounds from the same plant extracts or mixture of them (Wagner, 1999). The previous findings of classical pharmacology with mixtures of bioactive compounds have shown that we have to differentiate between additive and synergistically acting overadditive or potentiating effects. If two substances of a mixture have the same pharmacological target, an additive effect may be expected. However, if two or more substances of a mixture have different pharmacological targets, a synergistic effect may result, which can be greater than expected for the individual substances taken together. Dose-dependent investigation with mixtures of bioactive compounds can be carried out by using the isobologram methods, as proposed by Berenbaum (1989). In an experiment performed using the thrombocyte aggregation assay with a mixture of Gingolides A and B, two major constituents of *Ginkgo biloba*, a typical synergistic effect was shown by a 'concave up' isobologram curve (Steinke, 1993).

In studying the possible molecular mechanisms of synergistic therapeutic effects, it is crucial to take into consideration that a part of these effects can also be due to an

enhanced absorption or excretion rate and better bioavailability caused by nonbioactive constituents of the same herbal drug, such as tannins or saponins (Wagner, 1999).

2.5 Major groups of antimicrobial compounds from plants

Plants have a limitless ability to synthesise aromatic substances, most of which are phenolics or their oxygen-substituted derivatives (Gelssman, 1963). Most are secondary metabolites, of which at least 12,000 have been isolated (Schultes, 1978). In many cases, these substances serve as the plants' defence mechanism against predation by microorganisms, insects and herbivores (Cowan, 1999). Useful antimicrobial phytochemicals can be divided into several categories as described below and summarised by table 2.2.

2.5.1 Phenolics and polyphenols

The phenolics or polyphenol groups include a wide range of plant substances having in common an aromatic ring with one or more hydroxyl groups and they tend to be soluble in water as they occur in combined forms with sugars as heterosides. These are described as the most stable biochemicals and the most widely distributed secondary metabolites found in every family and in practically every species screened for their presence so far (Anon., 2004).

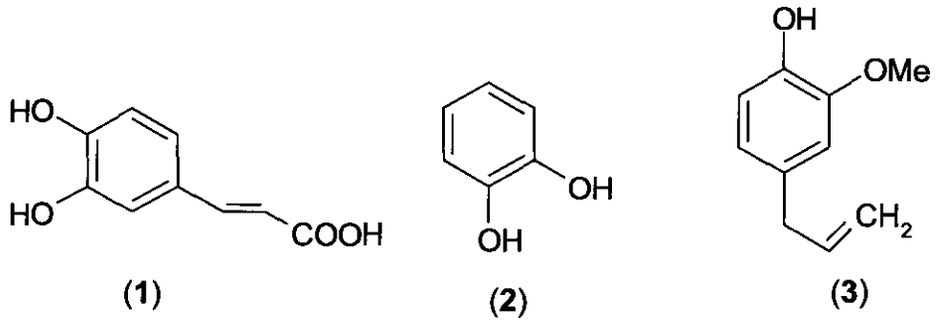
2.5.1.1 Simple phenolics and phenolic acids

Some of the simplest bioactive phytochemicals consists of a singly substituted phenolic ring. Most of the simple phenols are monomeric compounds of polymeric polyphenols and acids, which make up plant tissues, including lignin, melanin, flavolan and tannins. The common representatives of a wide group of phenylpropane-derived compounds, which are in the highest oxidation state, are cinnamic acids and caffeic acids (**1**). Caffeic acid which is found in common herbs such as tarragon and thyme is reported to be active against viruses, bacteria and fungi (Cowan, 1999). Other phenolics with antimicrobial activity include catechol (**2**) and eugenol (**3**). In some studies (Gelssman, 1963), it was also shown that the site(s) and number of hydroxy groups (-OH) on the phenol group have crucial correlation with relative toxicity to microorganisms.

Table 2.2: Major classes of antimicrobial compounds (Adapted from Cowan, 1999).

Phenolics	Simple phenols	Catechol	Substrate deprivation
		Epicatechin	Membrane disruption
	Phenolic acids	Cinnamic acid	
	Quinones	Hypericin	Bind to adhesins, complex with cell wall, inactivate enzymes
	Flavonoids	Chrysin	Bind to adhesins
	Flavones		Complex with cell wall
		Abyssinone	Inactivate enzymes
			Inhibit HIV reverse transcriptase
	Flavonols	Totarol	unknown
	Tannins	Ellagitannin	Bind to proteins
			Bind to adhesins
			Enzyme inhibition
			Substrate deprivation
			Complex with cell wall
			Membrane disruption
			Metal ion complexation
	Coumarins	Warfarin	Interaction with eucaryotic DNA (antiviral activity)
Terpenoids, essential oils		Capsaicin	Membrane disruption
Alkaloids		Berberine	Intercalate into cell wall and/or DNA
		Piperine	
Lectins and polypeptides		Mannose-specific agglutinin	Block viral fusion or adsorption
		Fabatin	Form disulfide bridges
Polyacetylenes		8S-Heptadeca-2(Z),9(Z)-diene-4,6-diyne-1,8-diol	unknown

As the degree of hydroxylation on the phenolic group increases, evidence exist that the toxicity also increases (Gelssman, 1963). An increase in the degree of oxidation on phenol was also reported to lead to increased inhibitory effects on microbes (Scalbert, 1991). The mechanism of action of phenolics against microorganisms include enzyme inhibition by oxidised compounds (Mason & Wasserman, 1987).



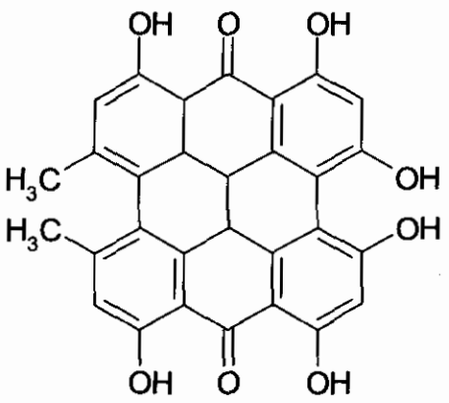
(1) (2) (3)

Figure 2.1: Phenolics and phenolic acid with antimicrobial activity.

2.5.1.2 Quinones

Chemically, compounds possessing either a 1,4-diketocyclohexa-2,5-dienoid or 1.2-diketocyclohexa-3,5-dienoid moiety (fig. 2.3) are classified as quinones. In the former case they are named p-quinones and in the latter case are named o-quinones. Most natural occurring quinones are p-quinones while the o-quinones are less common (Leistner, 1981). Quinones are ubiquitous in nature and are characteristically highly reactive (Schmidt, 1988). They form strongly coloured pigments covering the entire visible spectrum. They are however, usually found in the interior regions of the plant and thus don't impart colour to the exterior of the plant. The structure of natural occurring quinones is based on the benzoquinone (**6**), naphthoquinone (**7**) or anthraquinone (**8**) ring system. These compounds are reported to be responsible for the browning reaction in cut or injured fruits and vegetables (Schmidt, 1988). Quinones such as quinone (**5**) are also reputed for their biological properties including antimicrobial activity. Anthraquinone from *Cassia italica*, was reported to be bacteriostatic against *Bacillus anthracis*, *Corynebacterium pseudodiphthericum*, and *Pseudomonas aeruginosa* and bactericidal for *Pseudomonas pseudomalliae* (Kazmi *et al.*, 1994).

Another well-known anthraquinone that has antimicrobial activity is hypericin (**4**) from St. John's wort (*Hypericum perforatum*) (Duke, 1985). Quinones are known to complex irreversibly with nucleophilic amino acids in proteins, often leading to inactivation of proteins and loss of function. This may be the mode of antimicrobial action of the quinones. The probable targets of these quinones in the microbial cell are surface-exposed adhesions, cell wall polypeptides and membrane-bound enzymes. Quinones may also act on microorganisms by rendering their substrate unavailable and thus leading to cell death (Cowan, 1999).



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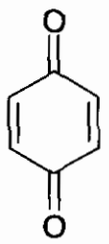

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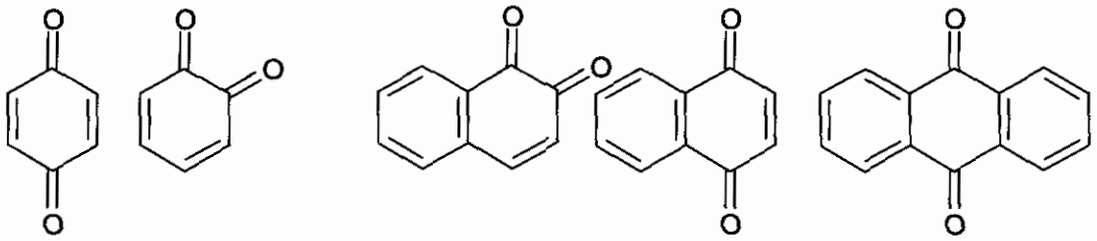
Figure 2.2: Quinones with antimicrobial activity.



(6) (7) (8)

Figure 2.3: Basic structures of naturally occurring quinones.

2.5.1.3 Tannins

The tannins are common to vascular plants existing primarily within woody tissues. They are oligosaccharide compounds, which consist of various phenolic compounds that react with proteins to form water insoluble copolymers. They are soluble in water with the exception of some high molecular weight structures. Plant tissues that are rich in tannins have a bitter taste and are avoided by feeders. Tannins may be classified as being either condensed or hydrolysable. Hydrolysable tannins such as pentagalloylglucose (**9**) are derived from gallic acid and they contain ester linkages that

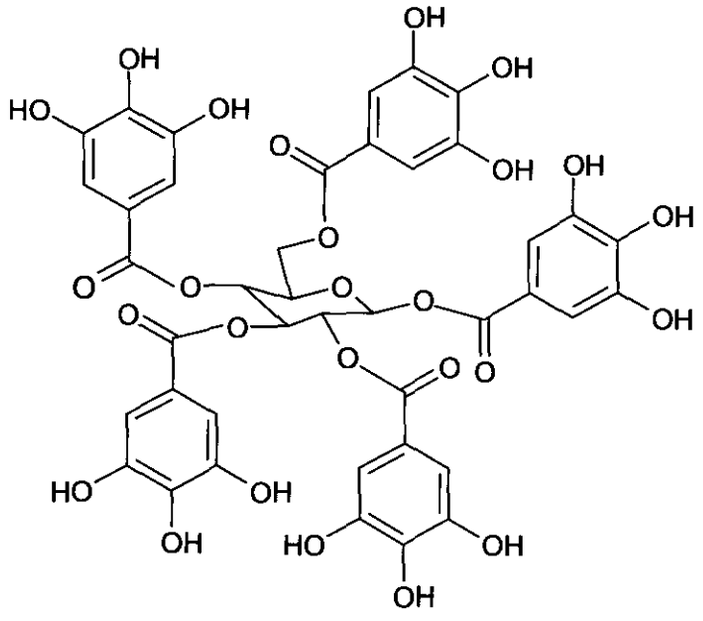
may be hydrolysed by hot water, mild acids or mild bases or an enzyme called tannase (under the same conditions condensed tannins do not hydrolyse), while more numerous condensed tannins (often called proanthocyanidins) as procyanidine –2 (**10**) are derived from flavonoid monomers (Robinson, 1983). Both procyanidine –2 (**10**) and pentagalloylglucose (**9**) have antimicrobial activity.

Hydrolysable tannins are often complex mixtures containing several different phenolic acids esterified to different positions of the sugar molecule (generally D glucose). For example tannic acid is usually a mixture of free gallic acid and various galloyl esters of glucose. Depending on the phenolic groups esterified to the core sugar, hydrolysable tannins can be classified as gallotannins (contain gallic acid) or allagitannins (contain ellagic acids as core). Some authors also define additional classes of hydrolysable tannins: taragallotannins (gallic acid and quinic acid as a core) and caffetannins (caffeic acid and quinic acid as core). Hydrolysable tannin molecules are usually composed of a core of D-glucose and 6 to 9 galloyl groups (Anon., 2004). These hydrolysable tannins are usually amorphous, hygroscopic, yellow to brown substances that dissolve in water (especially hot) to form colloidal rather than true solutions. The purer they are, the less soluble they are in water and the more readily they are obtained in a crystalline form. They are to some extend soluble in polar organic solvents, but not in non-polar organic solvents like benzene and chloroform. Hydrolysable tannins may be precipitated from aqueous solution by mineral acids or salts (Robinson, 1983).

Proanthocyanidins (condensed tannins) are oligomers or polymers of flavonoid units (i.e. flavan-3-ol) linked by carbon-carbon bonds not susceptible to cleavage by hydrolysis. The term, proanthocyanidins is derived from the catalysed oxidation reaction that produces red anthocyanidins upon heating proanthocyanidins in acidic alcoholic solutions. Proanthocyanidins may contain from 2 to 50 or more flavonoid units. Depending on their chemical structure and degree of polymerisation, proanthocyanidins may or may not be soluble in aqueous organic solvents.

Tannins can also be formed by polymerisation of quinone units. Many physiological activities such as anti-infective action have been associated with tannins (Cowan, 1999). In 1991, Scalbert pointed out that tannins could be toxic to filamentous fungi, yeast and bacteria. One of the mechanisms of their action is thought to be through formation of complexes with proteins through the hydrogen bonds and hydrophobic

effects, as well as covalent bonds. They can also complex with polysaccharides, but the antimicrobial significance of this effect has not been explored. Evidence also suggests that tannins can directly inactivate microbes, for example, low tannin concentrations modify the morphology of germ tubes of *Crinipellis perniciosa* (Brownlee *et al.*, 1990). There are speculations that tannins are at least partially responsible for the antimicrobial activity of the methanolic extract of the bark of *Terminalia alata* found in Nepal (Taylor *et al.*, 1996). On exposure to UV light (320 to 400nm at 5/m^2 for 2h) this activity of the methanolic extract of the bark of *Terminalia alata* was enhanced (Cowan, 1999).



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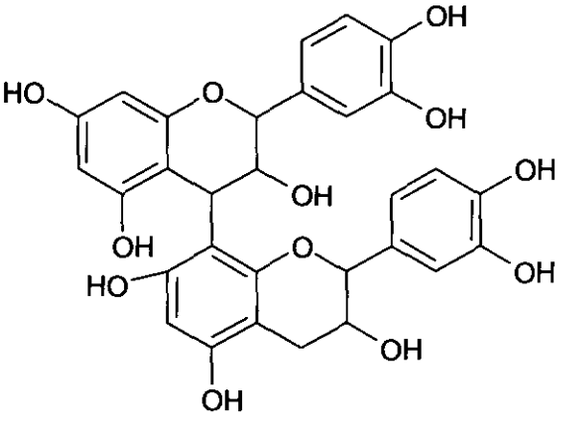


(10)

Figure 2.4: Tannins with antimicrobial activity.

2.5.1.4 Coumarins

The name coumarin is derived from the vernacular name (coumarou) of the tonka bean (*Dipteryx odorata)* from which coumarin (**11**) itself was first isolated in 1820 (Bruneton, 1999).

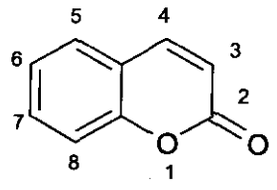


Figure 2.5: Basic nucleus of coumarins.

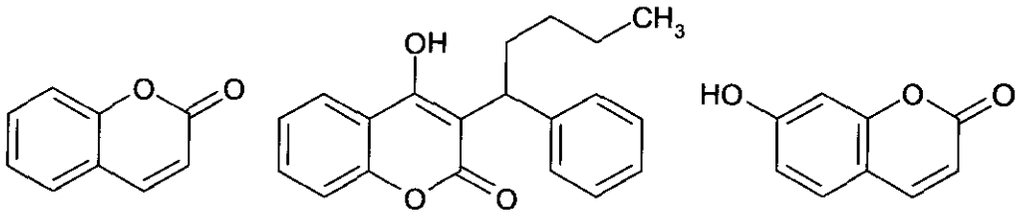
Coumarines are lactones of o-hydroxycinnamic acids and the basic nucleus with its numbering is as shown by the figure above and they belong to a group of compounds known as the benzopyrones, all of which consist of a benzene ring joined to a pyrone. Coumarine (**11**) and the other members of the coumarin family are benzo-α-pyrones, while other main members of the benzopyrone group –flavonoids contain the γ-pyrone group (Keating & O'kennedy, 1997). Coumarins have inherent fluorescent properties. They are often roughly categorised on the basis of their structures as follows (Murray *et al.*, 1982):

- Simple coumarines - these are the hydroxylated, alkoxylated and alkylated derivatives of the parent compound, coumarine, along with their glycosides.
- Furanocoumarines - these compounds consist of a five membered furan ring attached to the coumarin nucleus, divided to linear and angular types with substituents at one or both of the remaining benzenoid positions.
- Pyranocoumarines - members of this group contain pyrone instead of furan.
- Coumarins substituted in the pyrone ring.

In nature coumarins may be found in combination with sugars as glycosides. Almost all naturally occurring coumarins have oxygen (hydroxyl or alkoxyl) at C-7, but other positions may also be oxygenated and alkyl side-chains are frequently present. Coumarins can also be artifacts which arise from enzymatic hydrolysis of glycosyl-O-hydroxycinnamic acids and immediate cyclisation of the lactone. For example, coumarin, which was reported to have antimicrobial properties, can arise from enzymatic hydrolysis of melilotosides (Robinson, 1983). Ring closure to the lactone occurs only with o-hydroxy-cis-cinnamic acids (coumarinic acids). Ortho-hydroxy-trans-cinnamic acids (coumaric acids) do not form lactones directly. However, isomerisation to the cis form can be brought about by irradiation with UV light, whereupon immediate ring closure occurs. On the other hand, the lactone ring of coumarins can be opened by hydrolysis with warm alkali, but immediately reforms on acidification (Robinson, 1983). Coumarins have various bioactivities including, antifungal, antibacterial, antimalarial and antiviral activities (Ojala, 2001). Coumarines with known antimicrobial activity include warfarin (**12**), 7-hydroxycoumarine (**13**) and coumarin (**11**). Inophyllums isolated from calophyllum, are inhibitors of RT (reverse transcriptase) and HIV (Human Immunodeficiency Virus) replication in cell cultures (Patil *et al.*, 1993). Coumarin was

found to be active against *Candida albicans in vitro* (Thornes, 1997). As a group, coumarins are reported to have stimulatory effect on macrophages, which could have an indirect negative effect on infection. Hydroxyl cinnamic acids, related to coumarins, seem to be inhibitory to Gram-positive bacteria (Cowan, 1999; Fernandez *et al.*, 1996).

Kayser and Kolodziej (1999) studied the structural requirements of coumarins for antimicrobial activity. They found that while coumarins with the methoxy group at C-7 and if present, an OH group at either C-6 or C-8 are invariably effective against tested Gram-negative bacteria and a Gram-positive, *Staphylococcus aureus*. The presence of an aromatic demethoxy arrangement is apparently favourable against microbes which require special growth factors (beta-haemolytic *Streptococcus*, *Streptococcus pneumoniae* and *Haemophilus influenza*). The combination of these structural features, i.e. the methoxy groups and at least one phenolic group as reflected by the highly oxygenated coumarins, identify promising candidates with a broad spectrum of antibacterial activity. Although the primary site of synthesis in plants is suggested to be in the young, actively growing leaves, there is a possibility of species and compound variations. For example furanocoumarins in *Pastinaca sativa* are formed in the fruits were they accumulate while furanocoumarins in *Angelica archangelica* are formed in the leaves with the exception of osthenol, a simple coumarin, which is probably formed in the roots (Ojala, 2001).



(11) **(12)** **(13)**

Figure 2.6: Coumarins with antimicrobial activity.

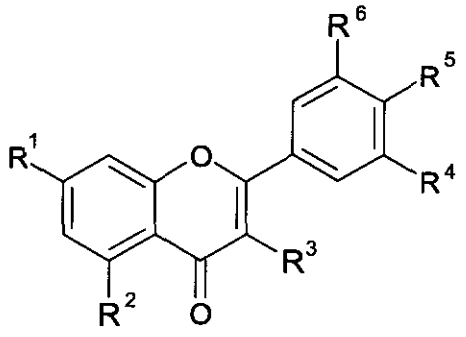
2.5.1.5 Flavonoids

The flavonoid group may be described as a series as C6-C3-C6 compounds. That is, their carbon skeleton consists of two C6 groups (substituted benzene rings) connected by a three-carbon aliphatic chain. There are different classes within which the groups are distinguished by additional oxygen heterocyclic rings and by hydroxyl groups

distributed in different patterns. The largest group of flavonoids is characterised by containing a pyran ring linking the three–carbon chain with one of the benzenes (Robinson, 1983).

The flavonoids are classified as secondary metabolites of low molecular weight, widely distributed in the plant kingdom, with several bioactivities, including antimicrobial activity (Hernandez *et al.*, 2000). Apigenin (**14**), a monohydroxylated flavone in the B ring, quercetin (**15**), a hydroxylated flavonol in the B ring and myricetin (**16**) trihydroxylated in ring B (Nishino *et al.*, 1987) are all reported to have antimicrobial activity. In other studies, five 5,6,7-trisubstituted flavones isolated from *Gomphrena martiana* and *Gomphrena boliviana* were found to have similar inhibitory effects on *Mycobacterium phlei* to commercial bactericides (Pomilio *et al.*, 1992). As some flavonoides are known to be synthesised in response to microbial infections, it should not be surprising that they are effective antimicrobial substances *in vitro* against a wide range of microorganisms (Cowan, 1999). This activity is thought to be attributed to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may disrupt microbial membranes (Tshuchiya *et al.*, 1996).

The description of the possible mechanism of action of flavonoides is hampered by conflicting findings. Flavonoids that are lacking hydroxyl groups on the B ring are more active against microorganisms than those with the –OH group. This finding supports the idea that their microbial target is the membrane, so lipophilic compounds would be more disruptive of this structure (Cowan, 1999). Some studies, however, show that the more hydroxylation, the greater the antimicrobial activity (Sato *et al.*, 1996). It is therefore safe to say that there is no clear predictable correlation between the degree of hydroxylation and antimicrobial activity (Cowan, 1999).



(**15**) R^1= OH, R^2 = OH, R^3= H, R^4= H, R^5= OH, R^6 = OH

(**16**) R^1= OH, R^2 = OH, R^3= H, R^4= OH, R^5= OH, R^6 = OH

(**14**) R^1= OH, R^2 = OH, R^3= H, R^4= OH, R^5= H, R^6 = H

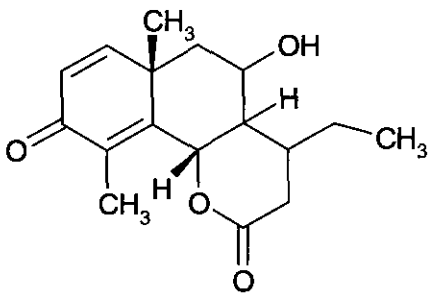
Figure 2.7: Common flavonoids with antimicrobial activity.

2.5.2 Terpenoids and essential oils

Medicinal and aromatic plants produce a wide variety of volatile terpene hydrocarbons (aliphatic and cyclic) and their corresponding oxygenated isoprenoid derivatives and analogues. A mixture of these substances, which are known as essential oils, can be isolated from diverse parts of plants by distillation (Magiatis *et al.*, 2001). Terpenoid structures are diverse and range from relatively simple linear hydrocarbon chains to highly complex ring structures. Cyclic terpenoids include monoterpenes (10 carbons) derived from geranyldiphosphate, sesquiterpenes (15 carbons) and triterpenes derived from farnesyldiphosphate and diterpenes (20 carbons) derived from gerenlygerenly diphosphate. The compounds comprise an especially important class of compounds in plants, as they mediate plant-plant, plant-insect and plant-pathogen interactions (Back & Chappell, 1996).

From a health perspective, these compounds represent important classes of antimicrobial and chemotherapeutic agents (Back & Chapell, 1996). Artemisinin (**17**), menthol (**18**) and capsaicin (**19**) were showed to have antimicrobial activity. The chemical composition of the essential oil of the resurrection plant *Myrothamnus flabellifolius* was determined in 2002. The terpenoids, pinocarvone and *trans*-pinocarvone were found to be the major compounds of the essential oil from this plant and were found to be responsible for the antimicrobial activity of this essential oil (Viljoen *et al.*, 2002).

In 1977, it was reported that 60% of the essential oil derivatives examined then were inhibitory to fungi while 30% inhibited bacteria (Chaurasia & Vyas, 1977). The mechanism of action of terpenes is not fully understood, but it is speculated to involve membrane disruption by the lipophilic compounds (Cowan, 1999). The ethanol soluble fraction of purple prairie clove (*Petalostemum purpureum*) yields a terpenoid called petalostemumol, which showed excellent activity against *Baccilus subtilis*, and *Staphylococcus aureus* and lesser activity against Gram-negative bacteria as well as *Candida albicans* (Hufford *et al.*, 1993).



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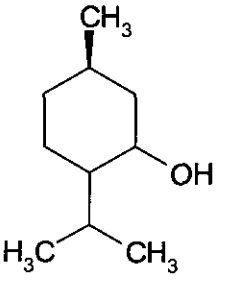

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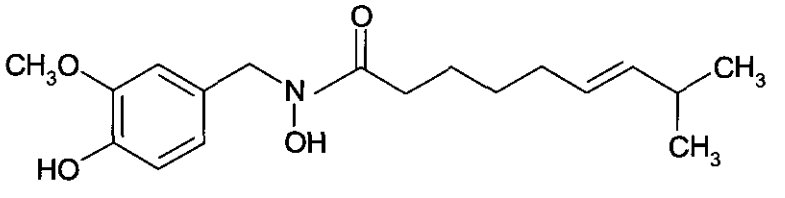


(19)

Figure 2.8: Terpenoids with antimicrobial properties.

2.5.3 Lectins and polypeptides

The term peptides include a wide range of compounds varying from low to very high molecular weights and showing marked difference in physical, chemical and pharmacological properties. These more or less complex compounds have two or more amino acid molecules united by a peptide linkage which result from elimination of water. On the other hand, lectins are proteins that interact specifically with carbohydrates. Many of them have the property of agglutinating red blood cells and are therefore also

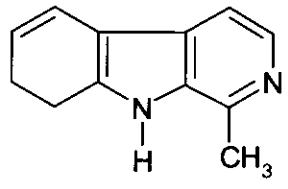
called hemoglutinins. Lectins usually have molecular weights above 100,000, contain ions (Mn^{2}+and Ca^{2}+) and many have as much as 50% carbohydrates (Van Wauwe *et al.*, 1973). The biological activity of the lectins may be attributed to the metal ions, which are the essential part of the native structure of most leguminous lectins. Lectins are reported to play an important role in defense mechanisms of plants against attacks by microorganisms, pests and insects. Fungal infections or wounding of the plants seems to increase lectin content (Bell, 2003).

Many suggestions have been made regarding the possible functions of lectins, which include protection against bacteria or insects and chemotactic agents for nitrogen-fixing bacteria (Robinson, 1983). Balls *et al.* reported peptides with inhibitory effects on microorganisms in 1942 and they are often positively charged and contain disulfide bonds (Zhang & Lewis, 1997). Thionins are peptides commonly found in barley and wheat, which consist of 47 amino acid residues. They are reported to be toxic to yeast, Gram-negative and Gram-positive bacteria (Cowan, 1999). Their mechanism of action may be the formation of ion channels in the microbial membrane or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors. Mannan-binding lectin binds to specific carbohydrate structures on the surface of a range of microorganisms including bacteria, yeast, parasitic protozoa, and viruses, and has been found to exhibit antimicrobial activity mediated by killing terminal, lytic complement components or by promoting phagocytosis (Thiel, 1998). α-(1-3)- and α-(1-6)-D-mannose-specific plant lectins were reported to be markedly inhibitory to human immunodeficiency virus and cytomegalovirus infections *in vitro* (Cowan, 1999). It should be emphasized that molecules and compounds such as these whose mode of action may be to inhibit adhesion will not be detected by most general plant antimicrobial screening protocols, even with the bioassay-guided fractionation procedures (Lewis & Elivin-lewis, 1995).

2.5.4 Alkaloids

Alkaloids are nitrogen-containing heterocyclic compounds which occur mainly in plants as their salts of common carboxylic acids such as citric, lactic, oxalic, acetic, malic and tartaric acids as well as fumaric, benzoic, aconitic and veratric acids (Robinson, 1983). Many alkaloids are crystalline substances which unite with acids to form salts. In addition to the element carbon, hydrogen and nitrogen most alkaloids contain oxygen. A few such as coniine and nicotine are oxygen-free and are liquids. Although most

alkaloids are not coloured, coloured alkaloids such as berberine (**20**; yellow) also occur in nature. Knowledge of the solubility of alkaloids and their salts is of crucial pharmaceutical importance. In most cases, free bases of alkaloids are sparingly soluble in water but soluble in organic solvents while the opposite is the case with their salts. This difference in solubility between alkaloids and their salts provide methods for the isolation of alkaloids from plants and their isolation from non-alkaloidal substances. Of course there are some exceptions to the abovementioned solubility rule. For example, caffeine (base) is readily extracted from tea by water and colchicine is soluble in either acid, neutral or alkaline water (Trease & Evans, 1972).



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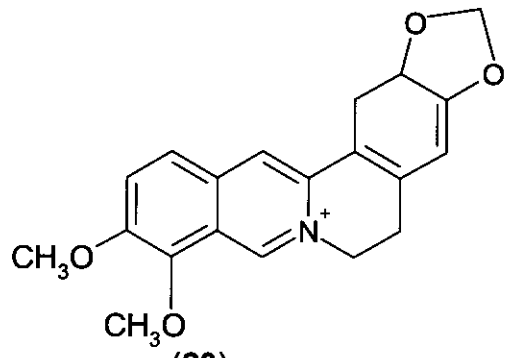

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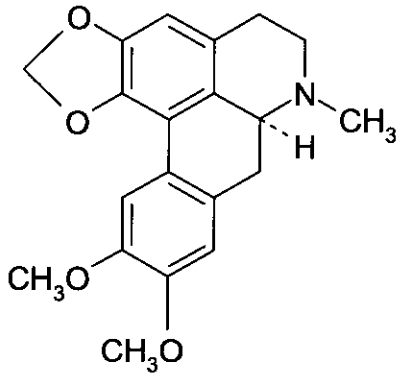


(21)

Figure 2.9: Alkaloids with antimicrobial activity.

Alkaloids are renowned for their potent pharmacological activities including antimicrobial activities. Chakrabortya *et al.* (1995) isolated carbazole alkaloids with antimicrobial activity from the leaves of *Clausena heptaphylla*. This alkaloid was found to be active

against both Gram-positive and Gram-negative bacteria and fungi. Plant derived alkaloids can even be more effective against microorganisms than some of the microorganism derived antibiotics. In one study the activity of berberine (**20**) exceeded that of chloramphenicol (e.g. Chloromycetin) against *Staphylococcus epidermidis*, *Neisseria meningitidis*, *Escherichia coli* and other bacteria (Dweck, 2003). Dicentrine (**21**), harmine (**22**) and several related alkaloids were also shown to have bactericidal activity. The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine (**20**) and harmane (**22**) is attributed to their ability to intercalate with bacterial DNA (Phillipson & O'Neill, 1987).

2.6 Approaches to drug discovery using higher plants

Many approaches are employed in the search for new biologically active principles in higher plants (Farnsworth & Loub, 1983) depending on the availability of information related to the source and resources. It is always crucial to make the best use of available information to avoid unnecessary waste of time, effort and resources. Some of the approaches are briefly discussed below.

2.6.1 Random selection followed by chemical screening

Phyotchemical screening approaches (i.e. for the presence of cardenolides/bufadenolides, alkaloids, triterpenes, flavonoids, isothiocyanates, iridoids, etc.) have been employed previously and are currently mainly pursued in the developing countries. Although the tests are simple to perform, they sometimes show false-negative and false-positive results, and thus render the results more difficult to assess. The other important fact is that it is usually impossible to relate one class of phytochemicals to specific biological targets; for example alkaloids or flavonoids produce a vast array of biological effects that are usually not predictable in advance (Fabricant & Farnsworth, 2001).

2.6.2 Random selection followed by one or more biological assay

In this approach, readily available plants are collected and extracts thereof are tested for one or more types of pharmacological activity. This random collection, broad screening method is contingent on the availability of sufficient funds and appropriate predictable bioassay systems.

Gordon H. Svoboda discovered vincristine, when he submitted an extract of the Madagascan periwinkle plant [C*atharanthus roseus* (L) G.Don] to a pharmaceutical screening program at Lilly. C*atharanthus roseus* was the fortieth plant he selected for inclusion in the screening program (Farnsworth, 1982). Today vincristine is the drug of choice for treatment of child leukemia while vinblastine is a secondary drug for treatment of Hodgkin's diseases and other neoplasms. The sulphates of these vinca alkaloids (vincristine sulphate and vinblastine sulphate) are the last useful drugs to reach the marketplace based on this procedure (Fabricant & Farnsworth, 2001).

2.6.3 Follow up of ethnomedical (traditional medicine) uses of plants

In this approach plants that are used to treat diseases in the traditional medicines are subjected to biological tests related to the diseases they are used for traditionally. Bioassay-guided methods are used to trace active principles. It was found that 84% of 119 chemical compounds used as drugs have the same or related use as the plants from which they were derived (Fabricant & Farnsworth, 2001). This shows that this approach is more reliable.

Ethnobotanical information can be acquired from different sources such as books on medicinal botany and herbals; review articles (usually involving surveys of medicinal plants by geographic region or ethnic culture); notes placed on voucher herbarium specimen by botanist at the time of collection; field work; and computer databases such as NAPRALERT and USDA-Duke (Fabricant & Farnsworth, 2001). Undocumented information carried from one generation to another in ethnic groups is rapidly disappearing as young members are drawn away from tribal life-style and oral traditions are not passed on. Mark Plotkin compared this loss of knowledge to the burning down of a library containing books that are one of a kind and irreplaceable (Kong *et al.*, 2003).

Chapter 3: Plants Selected for Screening

A literature study was conducted on plants traditionally used for the treatment of infection related diseases and plants were selected based on the guidelines suggested by Baker *et al.* (1995). According to these guidelines it is important to consider the following criteria when choosing a plant for investigating its medicinal and agrochemical potential:

- Evidence suggesting the traditional usage of the plant by native people;
- The purpose for which it is used;
- The abundance of the specific plant species in nature;
- Sustainable utilisation of the plant.

The seasonal and regional variations were not considered, although they could affect the potency of the extracts (Weenen *et al.*, 1990). The plants selected for the current study are as follows:

- *Antizoma angustifoli* (Burch.) Miers.
- *Carpobrotus acinaciformis* (L.)L.Bol.
- *Delosperm herbeum* (N.E.Br.) N.E.Br.
- *Melianthus comosus* (Vahl)
- *Physalis viscosa* L.
- *Rhus pyroides* (Burch)
- *Zanthoxylum capensis* (Thunb.) Harv.
- *Ziziphus mucronata* (Willd).

3.1 *Antizoma angustifolia* (Menispermaceae)

3.1.1 Description

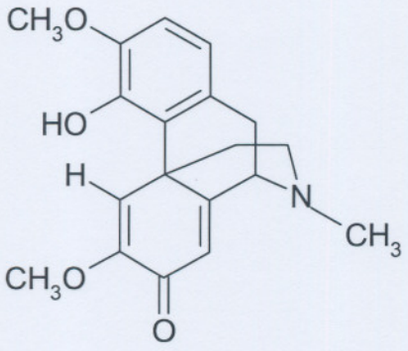
Figure 3.2: *Antizoma angustifolia.*

The genus *Antizoma* consists of only two species: *Antizoma angustifolia* and *Antizoma miersiana*. *Antizoma angustifolia* is an evergreen shrubby climber that normally grows in dry areas in Namibia, Botswana and the northern parts of South Africa (De Wet *et al.*, 2004). Species belonging to *Antizoma* are shrubs, scandent, prostrate or suberect.

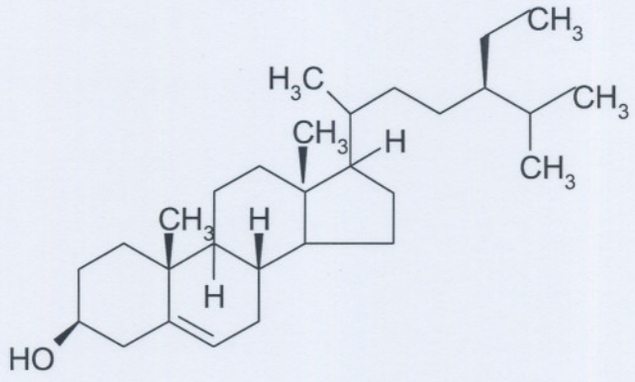
3.1.2 Chemical compounds isolated from *Antizoma* species

Chemicals isolated from *Antizoma angustifolia* include the following:

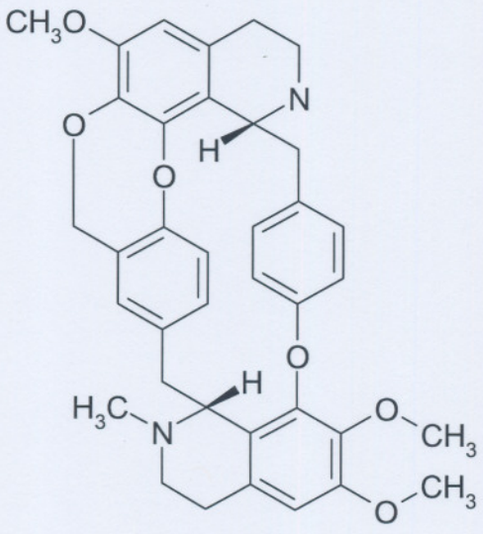
- proaporphine alkaloids - crotsparine (**28**), glaziovine (**29**) and pronuciferine (**30**)
- aporphine alkaloid - bulbocapnine (**27**)
- morphinane alkaloid- salutaridine/sinoacutine (**23**)
- bisbenzyltetrahydroquinoline alkaloids- cissacapine (**26**) and insularine (**25**)
- and phytosterol - β-sitosterol (**24**) (De Wet *et al.*, 2004; Dekker *et al.*, 1988).



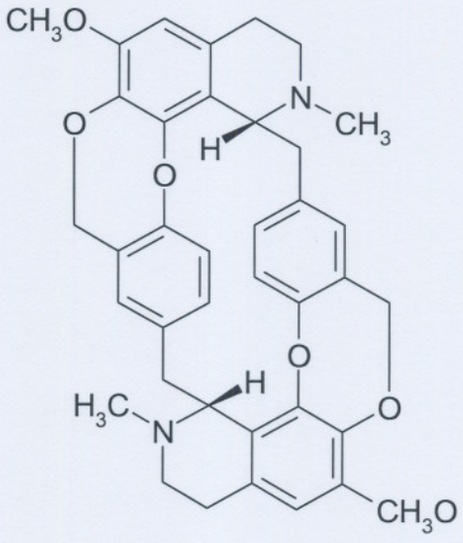
(**23**)



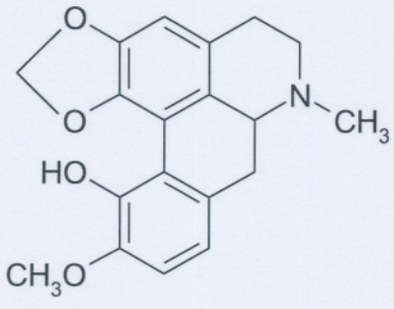
(**24**)



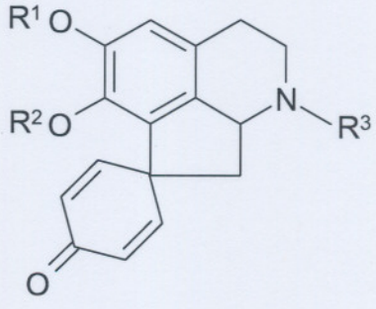
(**25**)



(**26**)



(**27**)



(**28**) R^1= CH_3, R^2 = R^3= H
(**29**) R^1= R^3 = CH_3, R^2= H
(**30**) R^1= R^2= R^3

Figure 3.1: Compounds isolated from *Antizoma genus*.

Sinoacutine has slight anti-inflammatory effects. *Antizoma capensis* was reported to contain an alkaloid called cissampeline (Watt & Breyer-Brandwijk, 1962).

3.1.3 Traditional uses of *Antizoma* species

Different tribes use *Antizoma species* for the treatment of different conditions, which include stomach ailments (such diarrhoeae and stomachache), pains, wounds and cough (Von Koenen, 2001; Watt & Breyer-Brandwijk, 1962). Dilute decoctions of the root of *Antizoma capensis* is taken as a blood purifier for boils and syphilis (Watt & Breyer-Brandwijk, 1962). Both the Africans and Europeans in the Prieska district use the powdered root of *Antizoma* species for the treatment of diarrhoea. In the western region of Cape Town, an extract of *Antizoma capensis* is used for the treatment of bladder ailment. The paste of the leaf and a decoction of the root of *Antizoma capensis* are used to treat snakebites. The paste is applied directly to the wound while the decoction is taken orally (Watt & Breyer-Brandwijk, 1962).

3.2 *Carpobrotus acinaciformis* (Aizoaceae)

3.2.1 Description

Figure 3.4: *Carpobrotus acinaciformis.*

Carpobrotus acinaciformis is a xerophilous plant native to South Africa and widely naturalised on the coast of southern Italy (Piattelli & Impellizzeri, 1970). This plant belongs to a family that is considered as one of southern Africa's most diverse and

abundant plant families, but the least studied for medicinal potential (van der Watt & Pretorius, 2001).

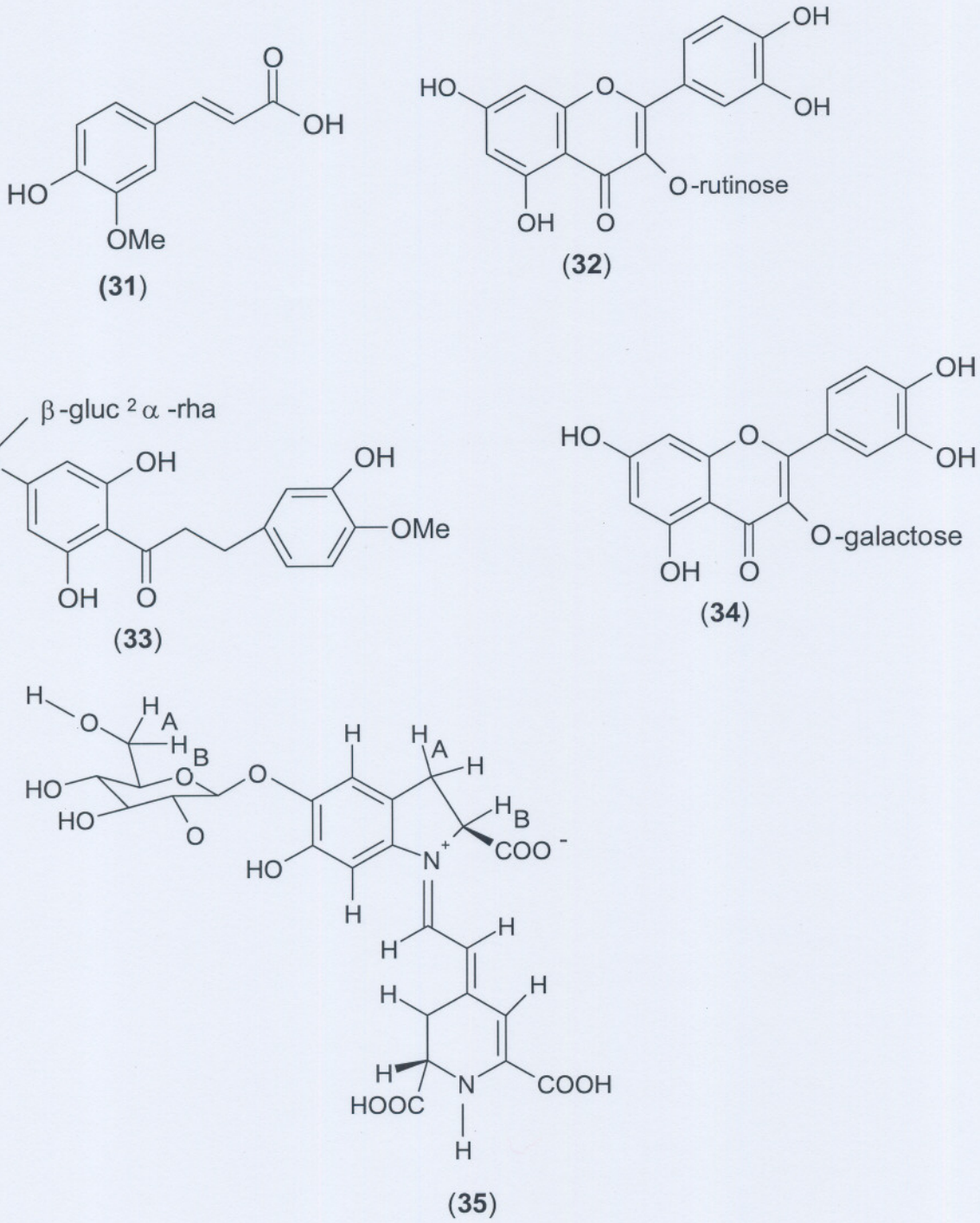


Figure 3.3: Structures of compounds isolated from *Carpobrotus* species.

3.2.2 Chemical compounds isolated from *Carpobrotus* species

The leaves of *Carpobrotus acinaciformis* have been reported to contain an alkaloid mesembrine, organic acids such as malic acid and citric acid and their calcium salts

(Watt & Breyer-Brandwijk, 1962). The purple flower of *Carpobrotus acinaciformis* have been found to contain, lampranthin-II, isolampranthin-II and a number of betacyanins including, 2-decarboxybetanidin, betanidin, betanin (**35**) and their epimers isobetanidin and isobetanin (Piattelli & Impellizzeri, 1970). In 2001 Van der Watt and Pretorius reported the isolation of five antimicrobial compounds, namely rutin (**32**), neohesperidin (**33**), hyperoside (**34**), cactichin and ferulic acid (**31**) from *Carpobrotus edulis.*

3.2.3 Traditional uses of *Carpobrotus* species

The leaves of *Carpobrotus* species have been very popular in the Cape as gargles for the treatment of throat infections (Smith *et al.*, 1998) and sore mouth (Watt & Breyer-Brandwijk, 1962). They are also used in the treatment of heart conditions (Piattelli & Impellizzeri, 1970). The boiled fruit of *Carpobrotus acinaciformis* was used for the treatment of pulmonary tuberculosis, other internal chest conditions, sore throat and sore mouth (Watt & Breyer-Brandwijk, 1962).

3.3 *Delosperma herbeum* (Aizoaceae)

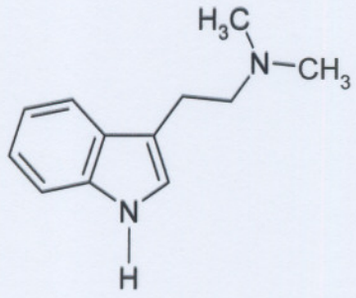
3.3.1 Description

Figure 3.6: *Delosperma herbeum.*

Delosperma is a genus of about 140 species of dwarf, succulent shrubs or biennial or perennial herbs, common in South Africa. *Delosperma herbeum* is one member of this genus commonly found in the Great Karoo rocky slopes in and it has white flowers (Slaby, 2002). It is a dainty looking groundcover succulent (Joffe, 2003).

3.3.2 Chemical compounds isolated from *Delosperma* species

Delosperma species were reported to contain dimethyltryptamine (**36**) and N-methyltryptamine (Smith, 1977).



(**36**)

Figure 3.5: Compound isolated from *Delosperma* species.

3.3.3 Traditional uses of *Delosperma herbeum*

The Tswana people use a decoction of the root of *Delosperma herbeum* in the treatment of the so-called climacteric and they believe that if they rub the powdered plant over the vertebral column it will to make them strong (Watt & Breyer-Brandwijk, 1962).

3.4 *Melianthus comosus* (Melianthaceae)

3.4.1 Description

Figure 3.7: *Melianthus comosus.*

Melianthus comosus is an attractive shrub with compound leaves and red, nectar-rich, bird pollinated flowers (van Wyk & Gericke, 2000). It is a multibranched shrub of up to 3 m in height. All parts of the plant produce a strong, unpleasant smell when touched or bruised. The leaves are clustered towards the tips of the branches. They are divided into about five pairs of leaflets which are oblong in shape, with prominently toothed margins. Small, bright red petals are borne in a short cluster, followed by a four-winged bladdery capsule (van Wyk *et al.*, 1997).

3.4.2 Chemical compounds isolated from *Melianthus comosus*

Melianthus comosus contains several toxic bufadienolides, of which meliathusigenin is a typical example. A triterpenoid, oleanic acid, as well as a cinnamic acid derivative thereof, has been isolated from the root bark (van Wyk *et al.*, 1997).

3.4.3 Traditional uses of *Melianthus comosus*

Leaf poultices and decoctions of *Melianthus comosus* are applied directly onto the impetigo, septic wounds, sores, ringworm, bruises, backache, and rheumatic joints. The dried and powdered leaf is applied directly to sore and open wounds and burns and is reported to relieve pains, retract the wounds, and facilitate healing (van Wyk & Gericke, 2000). A mixture of *Melianthus major, Lobostemon fracticosus, Cyanella lutea* and *Galenia africana* is made into an ointment for wounds especially on the legs of women. A remedy for syphilis is made from a mixture of *Lobostemon fructicosus, Melianthus comosus, Melianthus major and Galenia africana* (Watt & Breyer-brandwijk, 1962).

3.5 *Physalis viscose* (Solanaceae)

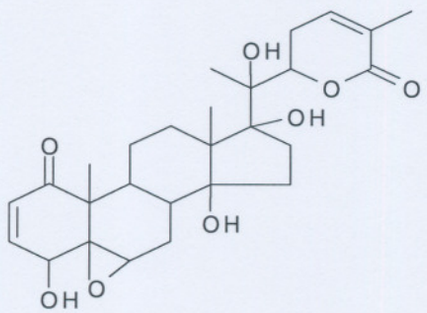
3.5.1 Description

Figure 3.9: *Physalis viscosa.*

Physalis viscosa (grape groundcherry) is a noxious perennial, which typically grows up to 0.6 m tall, with creeping roots. It is an erect, bushy or sprawling plant with more or less bell shaped greenish–yellow flowers and yellow berries enclosed in loose papery husks (enlarged calyx). This plant typical forms colonies. Foliages die back during the cold season and new growth is initiated in spring. Mature berries of *Physalis viscosa* are edible and are sometimes used in cooking or made into jam (CDFA, 2004).

3.5.2 Compounds isolated from *Physalis* species

The genus *Physalis* is known for elaborating complex structural variants of simple withanolides (Glotter, 1991). Withanolides are known for their broad spectrum of biological activity. The arial part of *Physalis viscosa* were found to contain withanolides such as 4ß-hydroxywithanolides E (**37**) and its 5,6-desoxi analogue, withaphysanolides (**38**), and withanolide related pregnanes such as 4ß-hydroxy-5ß,6ß-epoxypregn-2-ene-1,20-dione (**39**). The extracts from its root was also found to contain 4ß-hydroxywithanolide E (3) and withanolide D (**40**) (Silver *et al.*, 1993). The withanolides are a group of steroidal lactones, which have been isolated from the genera *Acnitus, Datura, Jaborosa, Lycium, Physalis* and *Withania* of family Solaniaceae (Glotter, 1991).



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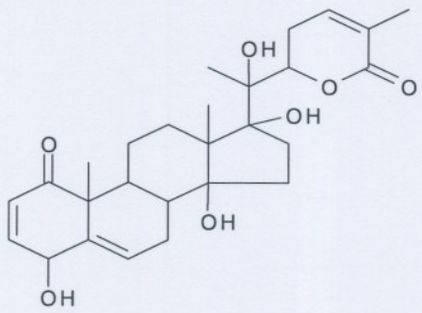

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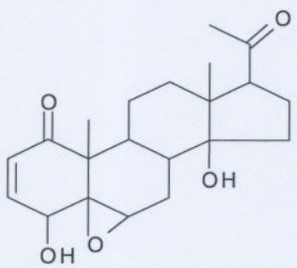

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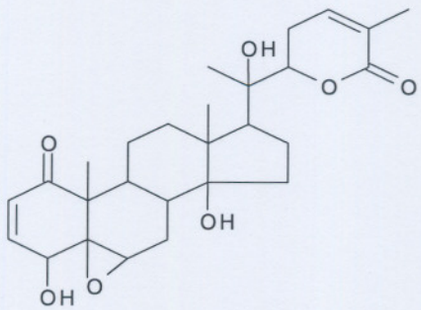


(40)

Figure 3.8: Compounds isolated from *Physalis viscosa*.

3.5.3 Traditional uses of *Physalis* species

Physalis peruviana is used as a diuretic and the juice of its leaves is given in worm and bowel complaints, while heated leaves are applied as a poultice (PID, 1969). *Physalis alkekengi* is used in Chinese medicine and is has expectorant, antitussive, diuretic and oxytocic activity (Basey *et al*, 1992).

3.6 *Rhus pyroides* (Anacardiaceae)

3.6.1 Description

Figure 3.11: *Rhus pyroides.*

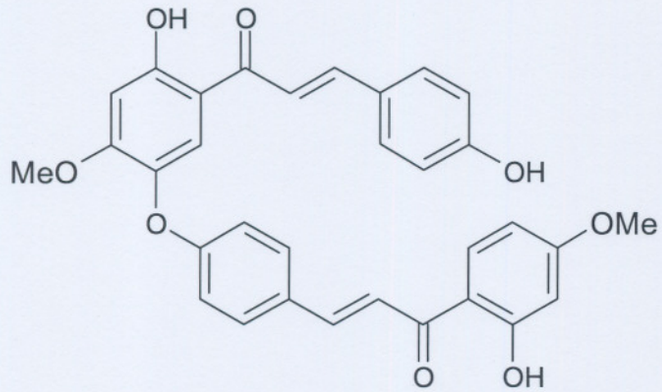
Rhus pyriodes is a shrub or small bushy to spreading tree, which has compound leaves with three obovate leaflets that are smooth or densely covered with hair, and the margins are either entire or irregularly toothed. It has very small yellow flowers arranged in terminal heads and its flowering time is from October to January (Germishuizen & FabianS, 1997).

3.6.2 Chemical compounds isolated from *Rhus* species

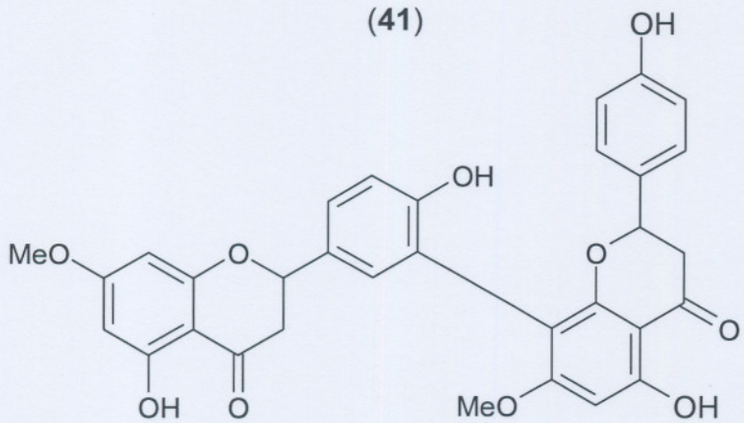
Compounds isolated from *Rhus pyroides* include bichalcone 2',4'',2'''-trihydroxy-4',4'''-dimethoxy-4-*O*-5'''-bichalcone also called rhuschalcone-1 (**41**) (Masesane *et al.*, 2000). *Rhus* species are reported to contain tannic acid in bark (10.15%), leaves (7.98 %) and twigs (Watt & Breyer-brandwijk, 1962).

The genus *Rhus* consist of approximately 200 species and is known to be rich in biflavonoids. Biflavonoids are very important due to various biological activities they manifest (Masesane *et al.*, 2000). Biflavonoids agathisflavone, robustflavone and hinokiflavone isolated from *Rhus succedanae* were found to have HIV-1 reverse transcriptase activity (Lin *et al.*, 1997). Ahmed *et al.* (2001) isolated the biflavanone (2S,2''S)-7,7''-di-O-methyltetrahydroamentoflavone (**42**) and five known flavonoids, 7-O-

methylnaringenin, 7,3'-O-dimethylquercetin, 7-O-methylapigenin, 7-O-methylluteolin, and eriodictyol from the leaves of *Rhus retinorrhoea.* The biflavanone (2S,2"S)-7,7"-di-O-methyltetrahydroamentoflavone exhibited moderate antimalarial activity with an IC_{50} value of 0.98 µg/ml against *Plasmodium falciparum* (W2 Clone) and weak activity against *P. falciparum* (D6 Clone) with an IC_{50} value of 2.8 µg/ml, but did not display any cytotoxicity. In addition, 7-*O*-Methylnaringenin showed weak antimicrobial activity against *Candida albicans, C. krusei, Staphylococcus aureus, Mycobacterium smegmatis, M. intracellulare, and M. xenopi* with a MIC (minimum inhibitory concentration) value of ~ 100 µg/ml (Lin *et al.*, 1997).



(**41**)



(**42**)

Figure 3.10: Some compounds from *Rhus pyroides.*

3.6.3 Traditional uses of *Rhus* pyroides

This plant plays an important role in traditional medicine, especially among the Kwena and Tswana tribes as they use its infusion as an eye lotion (Watt & Breyer-brandwijk, 1962).

3.7 *Zanthoxylum capensis* (Rutaceae)

3.7.1 Description

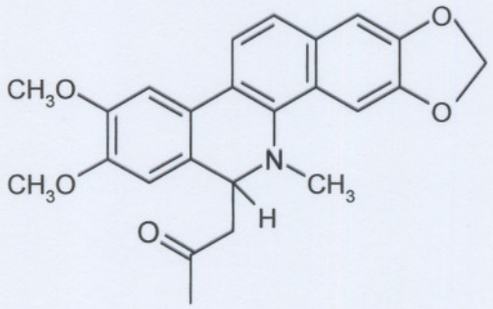
Figure 3.13: *Zanthoxylum capensis.*

Zanthoxylum capensis is a small much branched tree, usually about five meters tall but under favourable conditions it may grow to ten meters. The presence of thick thorns on the grey bark is a characteristic feature of the tree and common names all refer to this breast-like structure. Scattered, sharp thorns may be present on the stems. Leaves are divided into several pairs of leaflets, each about 20 mm in length, with translucent dots (oil glands) along the edges. The flowers are greenish–white and inconspicuous. Small orange–brown fruit of about 5 mm in diameter, resembling minute oranges, are produced in clusters (van Wyk *et al.*, 1997).

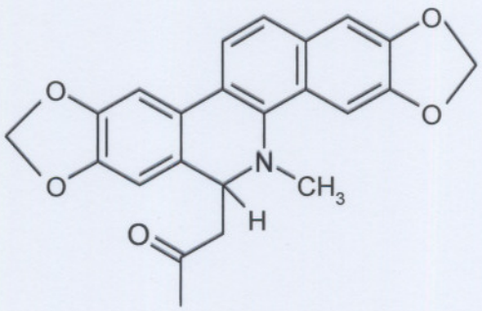
3.7.2 Chemical compounds isolated from *Zanthoxylum* species

This genus is known to elaborate a variety of biologically active secondary metabolites including alkaloids, lignans, terpenoids and coumarins (Gray, 1983). Compounds isolated from *Zanthoxylum* species include, 8-acetonyldihydronitinide, 8-acetonyldihydroavicine, liriodinine, savinine, sasamin, lichexanthone, (+)-piperitol-γ,γ-dimethylallylether, decarine and 8-O-desmethyl-*N*-nornitidine (Nissanka *et al.*, 2001). The two benzophenanthrene alkaloids, 8-acetonyldihydronitinide (**43**) and 8-acetonyldihyroavicine (**44**) have significant inhibitory effects on *Staphylococcus aureus* with MIC values of 1.56 and 3.12 μg/ml respectively, while liriodenine and savinine have moderate effects (Nissanka *et al.*, 2001). 8-acetonyldihydronitinideis and liriodinine were also found to have strong antifungal activity against *C. cladosporioides* (Nissanka *et al.*, 2001). In another study, Dieguez-Hurtado *et al.* (2003) screened *Zanthoxylum*

fagara, Z. elephantiasis and Z. martinicense for antifungal properties and found that the ethanolic extracts of the trunk bark showed activity against different species of fungi but no antibacterial activity was found.



(**43**)



(**44**)

Figure 3.12: Antimicrobial compounds isolated from *Zanthoxylum* Species.

3.7.3 Traditional uses of *Zanthoxylum* species

The pantropical genus *Zanthoxylum* has been credited with a range of ethnomedicinal properties. The specific species studied include those traditionally used for the treatment of diarrhoea, chest diseases, intermittent fever, earaches and tooth diseases (Roig, 1988; Gray, 1983). The early records showed that this traditional medicine was widely used, mainly for flatulence, colic, stomachache, fever and also for toothache and as a mouthwash (van Wyk *et al.*, 1997).

3.8 *Ziziphus mucronata* (Rhamnaceae)

3.8.1 Description

Figure 3.14: *Ziziphus mucronata.*

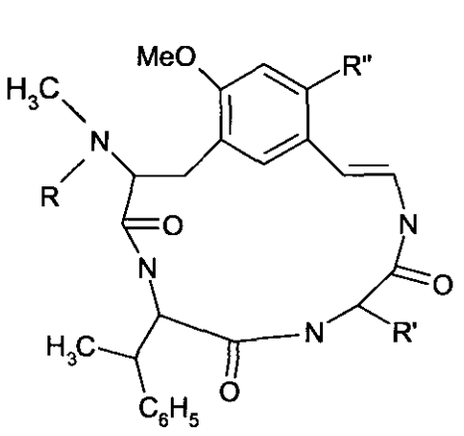
Ziziphus mucronata, also called buffalo thorn, is a small to medium-sized tree with a wide spreading crown and rough bark. Sharp thorns are present in pairs on the twigs – the one straight; the other curved. The leaves are bright green and shiny, with three main veins arising from the base. (van Wyk, & Gericke, 2000).

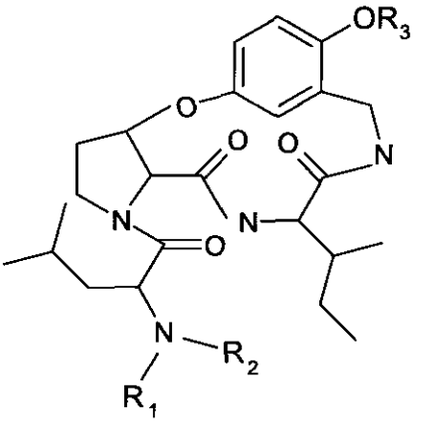
3.8.2 Chemical compounds isolated from *Ziziphus* species

Many *Ziziphus* species have been found to contain cyclopeptide alkaloids, which are particularly common in plants of Rhamnaceae (Tschesche & Kaussmam, 1975). Mucronines A-H have been isolated from bark of *Ziziphus mucronata* and they are all characterised by the presence of a styrylamine group in a 15-membered cyclopeptide alkaloid with the exception of Mucronine-D that is a 13-membered cyclopeptide alkaloid (Fehlhaber *et al.,* 1972: Tscheshe *et al.*, 1972: Tschesche *et al.*, 1974). In 1994, Barboni *et al.* reported the isolation of O-methylmucronine-D and known mucronine-D from the roots of *Ziziphus mucronata*. The bark of *Ziziphus abyssinica* was reported to contain abyssenine-A, abyssinine-B and abyssinine-C in addition to mucronine E-F. Mucronine-F.HCl, mucronine-G.HCl and mucronine-H.HCl from *Ziziphus mucronata* and Abyssenin-C from bark of *Ziziphus abyssinica* were reported to have weak antimicrobial activity (Tschesche *et al.*, 1974). *Ziziphus mauritiana* contains frangufoline, amphibines-B, -D, and –F together with mauritine A-F all of which belong to the same structural type with a 14-membered ring system containing *trans*-3-hydroxyproline, *p*-hydroxystryrylamine and one α-amino acid (Tschesche *et al.*, 1974).

3.8.3 Traditional uses of *Ziziphus mucronata*

This plant is much used in traditional medicine. The Africans in general apply a poultice of the leaf to boils, carbuncles and other septic swelling of the skin and the Zulus in particular also use its powdered leaves and barks in water as an emetic and for chest problems. The Batlhaping people take the infusion of the root for treatment of dysenteries and the Tongas chew the root and swallow the juice for the same purpose. This plant was also used in the treatment of gonorrhoea (Watt & Breyer-brandwijk, 1962).





	R	R'	R"
Mucronin-A (**45**)	CH_3	CH_3-C_6H_5	H
Mucronin-B (**46**)	H	CH_3-C_6H_5	H
Mucronin-C (**47**)	CH_3	$CH_2CH(CH_3)_2$	H
Mucronin-E (**48**)	CH_3	$CH_2CH(CH_3)_2$	OMe
Mucronin-F(**49**)	H	$CH_2CH(CH_3)_2$	OMe
Mucronin-G (**50**)	H	$CH(CH_3)C_2H_5$	OMe
Abyssenin-C (**51**)	H	$CH(CH_3)C_2H_5$	H

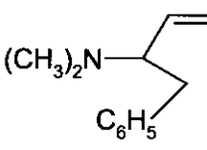
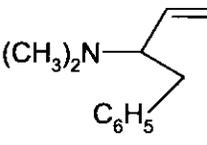
	R_1	R_2	R_3
Mucronin-D (**52**)	H		CH_3
O-demethy-mucronin-D (**53**)	H		H
New Compound (**54**)	CH_3	CH_3	CH_3

Figure 3.15: Compounds isolated from some *Ziziphus* species.

Chapter 4: Experimental and results

4.1 General experimental methods

4.1.1 Instrumentation

4.1.1.1 Nuclear magnetic resonance spectroscopy (NMR)

The ^{13}C and ^{1}H NMR spectra were recorded on a Varian Gemini-300 spectrometer. ^{13}C NMR spectra were recorded at 75,462 MHz while the ^{1}H NMR spectra were recorded at 300,075 MHz. The chemical shifts are reported in ppm (parts per million) relative to tetramethylsilane. The following abbreviations are used to describe the multiplicity of ^{1}H NMR signals: s = singlet, d = doublet, dd = doublet of doublets, ddd = doublet of doublet of doublets.

4.1.1.2 Infrared spectroscopy (IR)

The IR spectrum was recorded on a Nicolet Magna-IR 550 spectrometer, with the use of KBr pellets.

4.1.1.3 Mass spectroscopy

Mass spectra were recorded on an analytical Varian VG 7070E mass spectrometer using electron impact at 70 eV as ionisation technique.

4.1.2 Chromatographic techniques

4.1.2.1 Thin-layer chromatography (TLC)

Analytical TLC was performed on 0,25 mm thick silica gel aluminium backed sheets (Merk® TLC aluminium sheet silica gel 60 F_{254}). Chromatograms were examined under UV-light for the detection of the individual compounds. The chromatograms were also sprayed with 5% H_2SO_4 in ethanol to aid with visualization of poor chromophores.

4.1.2.2 Column chromatography

Column chromatography was performed with glass columns of different sizes. The stationary phase used was silica gel (Merk®; 0,063 - 0,063 mm).

4.2 Collection and storage of plant material

Plants were collected from the Potchefstroom area between February and April 2003 and identified by Mr. Bert Ubbinck and Prof. S. Cilliers from the Department of Botany, North-West University (Potchefstroom Campus). The plants were separated into morphological parts (leaves, roots, stems, etc.). This was done to determine in which part(s) the active principles responsible for antimicrobial activity are localised. The unnecessary plant material could then be disposed. Fleshy plants (*Carpobrotus acinaciformis* and *Delosperma herbuem*) were stored in the freezer until time of use when they were chopped into smaller pieces. The rest of the plant material was left at room temperature for 14 days to allow them to dry. These samples were then chopped into smaller pieces and stored in brown paper bags until time of use.

4.3 Preparation of extracts

The extracts were prepared using Soxhlet extraction. One of the advantages of Soxhlet extraction is that the sample is repeatedly brought into contact with fresh portions of the solvent. This procedure prevents the possibility of the solvent becoming saturated with extractable material and enhances the removal of the analyte from the matrix. Furthermore, the temperature of the system is close to the boiling point of the solvent providing energy in the form of heat that helps to increase the extraction kinetics of the system (Ganzler & Salgo, 1987). It is relatively inexpensive and selectivity of the extraction can easily be manipulated by altering the polarity of the solvent.

The disadvantages of Soxhlet extraction are that it requires several hours or days of extraction, the sample is diluted in large volumes of solvent and losses of compounds occur due to thermal degradation and volatilisation because of the heat supplied (Ganzler & Salgo, 1987).

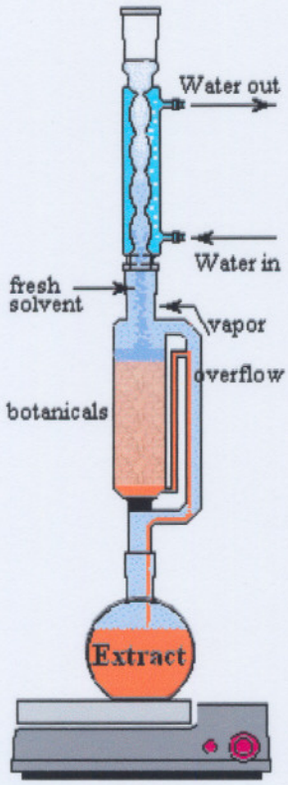


Figure 4.1: Soxhlet extraction apparatus (Amphora Society, 2003).

The plant material was filled into cellulose extraction thimbles and extracted by Soxhlet using solvents of varying polarity (Cannell, 1998). The following solvents were successively used, in order of increasing polarity:

- Petroleum ether (PE)
- Dichloromethane (DCM)
- Ethyl acetate (EtOAc)
- Ethanol (EtOH)

Increasing polarity.

This range of solvents was selected to reduce the complexity of extracts by selective extraction based on the polarity of phytochemicals. The plant material was extracted for 24 hours with each solvent (starting with the non-polar solvents), after which the extracts were concentrated *in vacuo* and allowed to dry completely in a fume hood.

4.4 Screening of plant extracts for antimicrobial activity

Screening of the selected plants was done in order to establish which of the plants exhibit definite antimicrobial activity. This procedure was crucial because further studies were conducted only on the plant that exhibited the best antimicrobial activity in the group of selected plants. The microorganisms used in this assay were Gram-positive *Bacillus subtilis* and *Staphylococcus aureus,* Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*, a fungus *Aspergillus niger* and a yeast *Candida albicans.* Two methods were used for determination of antimicrobial activity, namely the disc diffusion assay as described by van der Vijver and Lötter (1979) and the microplate method as described by Eloff (1998b).

4.4.1 Disc diffusion assay

The growth medium, composing of 16 g/ℓ nutrient broth (Rolab-Merck) and 12 g/ℓ bacteriological agar (Rolab-Merck), was sterilised for 15 minutes at 120 °C, allowed to cool down enough to hold by hand and poured into Petri dishes. 100 µl of 24 hour nutrient broth culture was spread evenly over the solid agar surface.

The dried plant extracts were reconstituted in 1 ml of acetone. Filter paper discs were soaked in these solutions for a few minutes, removed with tweezers and left to air-dry for an hour to allow evaporation of all solvents from the disks before use in the assay. The disks were placed onto the inoculated agar plates and incubated at 37 °C for 24 hours for bacteria, 48 hours for the yeast and 72 hours fungus. After incubation the plates were examined for zones of growth inhibition. The zones of inhibition were measured from the end of the disc to the end of the inhibition zone in millimetres. The results of this assay are depicted in table 4.1.

4.4.2 Minimum inhibitory concentration (MIC) determination for plant extracts

MIC was regarded as the lowest concentration of the compound or extract yielding no growth. The MIC for each extract was determined using a modification to the microplate method as described by Eloff (1998a).

In this method, colour change of *p*-iodotetrazolium violet (INT) was used to indicate the presence of living cells. The concentration of the extract in the first well without a colour change was regarded as MIC. The colour change is due to the reduction of the

colourless tetrazolium salt to a red formazan product by the action of NADH-generating dehydrogenases found in biologically active cells (Eloff, 1998a).

Table 4.1: Antimicrobial activity of screened plant extracts.

BOTANICAL NAME	PLANT PART	SOLVENT	TEST ORGANISMS					
			B.s.	S.a.	E.c.	P.a.	A.n.	C.a.
Antizoma angustifolia	Leaves	PE	2	-	I.C.	-	-	4
		DCM	3	1	1	-	-	3
		EtOAc	I.C.	-	2	-	-	1
		EtOH	1	9	2	-	-	3
	Root	PE	I.C	-	I.C.	-	-	1
		DCM	9	-	3	-	-	7
		EtOAc	4	1	1	-	-	3
		EtOH	7	2	1	-	-	4
Carpobrotus acinaciformis	Leaves	PE	-	I.C.	I.C.	-	-	-
		DCM	-	-	-	-	-	0,5
		EtOAc	-	-	-	-	-	-
		EtOH	-	-	-	-	-	-
	Twigs	PE	-	-	-	-	-	-
		DCM	-	-			-	1
		EtOAc	-	-	0,5	-	-	-
		EtOH	I.C.	-	I.C.	-	-	-
Delosperma herbeum	Leaves	PE	-	-	-	-	-	1
		DCM	-	-	4	1	-	1
		EtOAc	-	-	-	-	-	1
		EtOH	-	-	-	-	-	1
	Twigs	PE	2	-	1	-	-	1
		DCM	1	-	-	-	-	1
		EtOAc	I.C.	-	-	-	-	-
		EtOH	-	-	I.C.	-	-	-
Melianthus comosus	Leaves	PE	-	-	-	-	-	-
		DCM	-	-	-	-	-	-
		EtOAc	-	-	-	-	-	1
		EtOH	-	-	-	4	-	7
		MeOH	1		N.D.	2	-	2
		H_2O	2		N.D.	2	-	6
	Twigs	PE	-	-	-	-	-	1
		DCM	-	-	-	-	-	1
		EtOAc	-	-	-	1	-	-
		EtOH	-	-	-	-	-	-
	Seeds	PE	-	-	-	-	-	-
		DCM	-	-	-	-	-	-
		EtOAc	-	-	-	-	-	1
		EtOH	-	-	-	-	-	-
Physalis viscosa	Leaves	PE	-	-	I.C.	1	-	1
		DCM	-	-	-	6	-	-
		EtOAc	-	I.C.	-	4	-	1
		EtOH	-		-	6	-	I.C
	Twigs	PE	-	1	-	0,5	-	
		DCM	-	-	I.C.	-	-	
		EtOAc	I.C.	1	-	-	-	-
		EtOH	1	1	1	3	-	1
	Roots	PE	-	1	-	-	-	0,5
		DCM	-	1	-	4	-	0,5
		EtOAc	-	-	-	4	-	-
		EtOH	-	-	-	4	-	I.C.

BOTANICAL NAME	PLANT PART	SOLVENT	TEST ORGANISMS					
			B.s.	S.a.	E.c.	P.a.	A.n.	C.a.
Rhus pyriodes	Leaves	P.E	-	-	0,5	2	-	-
		DCM	-	-	-	2	-	-
		EtOAc	I.C.	-	-	2	-	-
		EtOH	-	-	-	3	-	-
	Twigs	PE	-	-	-	-	-	-
		DCM	-	1	-	-	-	I.C.
		EtOAc	-	I.C.	-		-	-
		EtOH	-	I.C.	-	2	-	-
Ziziphus mucronata	Leaves	PE	-	-	-	-	-	1
		DCM	1	-	-	-	-	1
		EtOAc	-	-	-	-	-	3
		EtOH	4	-	-	-	-	1,5
	Twigs	PE	-	-	-	-	-	1
		DCM	-	-	-	-	-	-
		EtOAc	-	-	-	-	-	-
		EtOH	-	-	-	-	-	-
	Bark	PE	-	-	-	-	-	-
		DCM	-	-	-	-	-	-
		EtOAc	-	-	-	0,5	-	1
		EtOH	-	-	-	-	-	-
Zanthoxylum capensis	Leaves	PE	-	-	-	-	-	-
		DCM	-	-	-	-	-	I.C.
		EtOAc	-	-	-	-	-	I.C.
		EtOH	-	-	-	-	-	2
	Twigs	PE	I.C.	-	-	-	-	3
		DCM	I.C.	-	-	-	-	3
		EtOAc	-	-	-	-	-	8
		EtOH	-	-	-	-	-	3

B.s. = *Bacillus subtilis*, S.a. = *Staphylococcus aureus*, E.c. = *Escherichia coli*, P.a. = *Pseudomonas aeruginosa*, A.n. = *Aspergillus niger*; C.a. = *Candida albicans*; numbers represent the size of the inhibition zones in mm; I.C. (Intrazonal colonies) = has some colonies within a zone of inhibition.

The cell cultures were standardised at the concentration of approximately 10^7 colony forming units/ml (cfu/ml). This cell culture standardisation is the modification made to the method by Eloff.

4.4.2.1 Preparation of extract solutions/suspensions

The plant extracts were suspended in 1 ml DMSO to prepare the relevant concentrations. The prepared concentrations were variable and ranging from 348 mg/ml to 1444 mg/ml. The concentrations were different because the prepared extracts of different weights were suspended in the same volume of DMSO.

4.4.2.2 Preparation of the microorganisms

The microorganisms used in this assay are Gram-positive *Bacillus subtilis* and *Staphylococcus aureus,* Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*, and a yeast *Candida albicans*. A fungus *Aspergillus niger* was excluded in this assay because it showed resistance to all extracts screened with the disc diffusion assay (table 4.1).

The microorganisms used were inoculated in 50 ml Mueller-Hinton (Fluka) broth at 37 °C and incubated for 24 hours for the bacteria and 48 hours for the yeast. Tween 80 (500 µl) (Merck) was added to *Candida albicans* and *Bacillus subtilis* broth cultures to ensure single cell suspensions. These broth cultures were diluted with sterile Mueller-Hinton broth to contain approximately 10^7 cfu/ml. The dilution was monitored by measuring the absorbance at 500 nm with a spectrophotometer (Milton Roy Spectronic 1201) to ensure that they contain appropriate cell concentrations (table 4.2; Swart, 2000).

Table 4.2: Absorbance values of different microorganisms at 500 nm used to prepare stock cultures.

Microorganism	Absorbance (nm)
S. aureus	0.030
E. coli	0.120
C. albicans	0.150
B. subtilis	0.120
P. aeruginosa	0.045

4.4.2.3 Preparation of microtiter plates

The broth (100 µl) was pipetted into all microplate wells. Thereafter, 100 µl of the prepared extract was added to the first set of wells and two-fold serial dilutions were made from column 1 to 11 in the microplate. In addition, 100 µl of stock culture was added to all columns except 11 and the plate was incubated for 24 hours at 37 °C.

After incubation, 20 µl of 0.2 mg/ml *p*-iodonitrotetrazolium violet [INT] (Sigma) was added to all the wells. With further incubation bacterial growth was indicated by a colour change to red. Column 11 served as 0% growth control (blank control) to give an indication of contamination, while column 12 served as the 100% bacterial growth control to ensure that normal growth occurs and INT accurately indicates growth.

Table 4.3: The MIC values (mg/ml) determined for crude plants extracts.

BOTANICAL NAME	PLANT PART	SOLVENT	PM	PEM	% Y	Test organisms				
						B. s.	S. a.	P. a.	E. c.	C. a.
Antizoma angustifolia	Leaves	PE	8,71	0,1521	1,75	152,1	152,1	152,1	152,1	152,1
		DCM		0,0205	0,24	0,32	10,25	10,25	1,28	1,28
		EtOAc		0,1965	2,26	49,1	N.D.	49,1	6,14	6,14
		EtOH		0,6056	6,95	151,4	302,8	302,8	2,37	2,37
	Roots	PE	3,73	0,013	0,35	>6,5	>6.5	>6,5	>6,5	>6,5
		DCM		0,012	0,32	12,1	12,1	12,1	> 6,0	> 6,0
		EtOAc		0,0177	0,47	> 8,5	> 8,5	> 8,5	> 8,5	> 8,5
		EtOH		0,0527	1,41	13,2	13,2	26,4	26,4	26,35
		H_2O		0,2539	6,80	N.D.	N.D.	N.D.	7,9	N.D.
Carpobrotus acinaciformis	Leaves	PE	47,67	0,1124	0,24	112,4	112,4	112,4	112,4	56,2
		DCM				N.D.	N.D.	N.D.	N.D.	5,0
		EtOAc		0,0466	0,10	11,17	46,6	23,3	23,3	23,3
		EtOH		0,0725	0,15	72,5	36,3	72,5	36,3	18,2
	Twigs	PE	47,77	0,707	1,48	353,5	353,5	353,5	88,4	88,4
		DCM				N.D.	N.D.	N.D.	N.D.	91.0
		EtOAc		0,627	1,32	162,7	162,7	20,3	81,4	40,7
		EtOH		0,2401	0,50	120,1	120,1	240,1	120,1	60,0
Delosperma herbeum	Leaves	PE	38,12	0,7452	1,96	93,2	372,6	372,6	372,6	46,6
		DCM		0,7225	1,90	722,5	180,6	45,2	360,1	361,3
		EtOAc		0,0293	0,08	29,3	29,3	29,3	29,3	7,3
		EtOH		0,1971	0,52	24,6	100,5	98,6	98,6	24,6
	Twigs	PE	37,08	0,1049	0,28	52,5	>104,9	>104,9	104,9	52,5
		DCM		0,5027	1,36	125,7	162,8	251,4	31,4	31,4
		EtOAc		0,3798	1,02	23,5	95,0	95,0	23,8	47,5
		EtOH		0,7244	1,95	45,3	362,2	181,1	>362,2	90,6
Melianthus comosus	Leaves	PE	12,73	0,3813	3,00	381,3	381,3	190,7	47,7	190,7
		DCM		0,0999	0,79	1,56	50,0	25,0	99,9	3,12
		EtOAc		0,075	0,59	9,4	37,5	75,0	ND	4,7
		EtOH		1,2204	9,59	4,8	610,2	19,1	610,2	152,6
		MeOH		N.D.	N.D.	N.D.	N.D.	40.9	N.D.	N.D.
		H_2O		N.D.	N.D.	N.D.	N.D.	45.0	N.D.	N.D.
	Twigs	PE	13,75	0,0514	0,37	51,4	51,4	25,7	51,4	25,7
		DCM		0,0644	0,47	16,1	64,4	16,1	16,1	16,1
		EtOAc		0,1016	0,74	50,8	50,8	6,3	50,8	12,7
		EtOH		0,3245	2,36	40,6	162,2	>162,2	162,2	20,3
	Seeds	PE	9,6	0,4032	4,20	>201,6	>201,6	201,6	>201,6	201,6
		DCM		0,4608	4,80	>461,0	>461,0-	>461,0	461,0	461,0
		EtOAc		0,0786	0,82	>393	>393	>393	78,6	39,3
		EtOH		0,4800	5,0	242,2	>484,3	484,3	484,3	120,1
Physalis viscosa	Leaves	PE		0,2718	2,6	68,0	>211,8	17,0	211,8	68,0
		DCM	10,5	0,2574	2,5	1,0	128,7	2,0	128,7	4,0
		EtOAc		0,2108	2,0	0,82	105,4	26,4	52,7	26,4
		EtOH		1,1759	11,20	18,4	147,0	18.4	73,5	18,4
	Twigs	PE	6,43	0,0338	0,52	> 33,8	8,5	8,5	33,8	33,8
		DCM		0,0263	0,41	13,2	13,2	13,2	13,2	13,2
		EtOAc		0,0464	0,72	46,4	11,6	46,4	11,6	23,2
		EtOH		0,3387	5,27	84,7	84,7	84,7	84,7	84,68
	Roots	PE	4,4	0,01	0,23	2,5	2,5	13,7	5,0	135,2
		DCM		0,0548	1,25	>54,8	27,4	13,7	27,4	54,8
		EtOAc		0,0605	1,38	15,1	> 60,5	15,1	> 60,5	60,5
		EtOH		0,4821	11,0	60,3	483,1	60,3	241,0	60,3

BOTANICAL NAME	PLANT PART	SOLVENT	PM	PEM	% Y	Test organisms				
						B. s.	S. a.	P. a.	E. c.	C. a.
Rhus pyriodes	Leaves	PE	12,6	0,5984	4,75	74,8	299,2	74,8	37,4	74,8
		DCM		0,1071	0,85	6,69	13,4	13,4	13,4	6,7
		EtOAc		0,1868	1,48	11,68	186,8	23,4	23,4	11,7
		EtOH		0,8596	6,82	429,8	214,9	107,5	53,8	214,9
	Twigs	PE	14,74		0,56	83,1	83,1	83,1	83,1	20,8
		DCM		0,0215	0,15	21,5	10,75	21,5	21,5	21,5
		EtOAc			0,49	36,3	36,3	36,3	36,3	18,13
		EtOH		0,4812	3,27	240,6	120,3	120,3	240,6	240,6
Ziziphus mucronata	Leaves	PE	15,21	0,0561	0,37	56,1	56,1	56,0	28,1	14,0
		DCM		0,1955	1,29	12,3	48,90	9,78	48,9	24,4
		EtOAc		0,4870	3.20	7,6	7,6	60,9	60,9	15,2
		EtOH		1,5224	10,0	190,3	>761,2	190,3	190,3	11,9
	Twigs	PE	10,98	0,055	0,50	55,0	55,0	27,5	55,0	72,5
		DCM		0,0401	0,37	20,1	40,1	5,0	20,1	5.0
		EtOAc		0,1295	1,18	64,8	64,8	8,1	64,8	64,8
		EtOH		0,2488	2,27	248,8	248,8	15,6	124,4	248,8
	Bark	PE	10,44	0,0016	0,015	>1.6	1,6	0,4	1,6	1,6
		DCM		0,1155	1,10	>57,75	11,5	11,5	11,5	11,5
		EtOAc		0,0605	0,58	30,3	>30,3	30,3	>30,3	15,1
		EtOH		0,086	0,82	>86,0	86,0	86,0	>86,0	86,0
Zanthoxylum capensis	Leaves	PE	14,84	0,1794	1,21	89,7	89,7	89,7	179,4	22,4
		DCM		0,1695	1,14	42,4	84,75	42,4	84,8	10,6
		EtOAc				N.D.	N.D.	N.D.	N.D.	N.D.
		EtOH		0,4925	3,32	492,5	492,5	246,3	246,3	30,8
	Twigs	PE	19,93	0,0582	0,29	58,2	58,2	58.2	58.2	29.1
		DCM		0,0854	0,43	42,7	42,7	42,7	85,4	21,3
		EtOAc		0,1735	0,87	17,35	17,35	86,8	86,8	2,7
		EtOH		0,2425	12,2	ND	121,3	121,3	242,5	2,0

B.s. = *Bacillus subtilis,* S.a. = *Staphylococcus aureus,* E.c. = *Escherichia coli,* P.a. = *Pseudomonas aeruginosa,* C.a. = *Candida albicans;* N.D. = not done. PM = Plant material mass (g); PEM = Plant extract mass (g), %Y = percentage yield.

After incubation, 20 µl of 0.2 mg/ml *p*-iodonitrotetrazolium violet [INT] (Sigma) was added to all the wells. With further incubation bacterial growth was indicated by a colour change to red. Column 11 served as 0% growth control (blank control) to give an indication of contamination, while column 12 served as the 100% bacterial growth control to ensure that normal growth occurs and INT accurately indicates growth.

Antizoma angustifolia DCM leaf extract showed a broad spectrum and best activity amongst tested extracts. As a result, *Antizoma angustifolia* leaf extracts were selected for further investigation.

4.5 Isolation and characterisation of compounds from *A. angustifolia*.

Antizoma angustifolia leaf extracts were fractionated using the activity-guided approach. The extracts were fractionated, fractions tested for antimicrobial activity and only active fractions were further fractionated to isolate the compounds responsible for their activity.

The DCM extract (3,58 g) of *A. angustifolia* leaves was fractionated on a silica gel column using petroleum ether:ethyl acetate (2:1) as a mobile phase. Eight major fractions were collected based on similarities in TLC. A fraction (fraction A) with a R_f value of 0,09 in petroleum ether:ethyl acetate (2:1) showed antimicrobial activity, when tested using the disc diffusion assay. This fraction was further chromatographed on a silica gel column using petroleum ether and ethanol mixtures in increasing polarity. Fraction A1 (2.1 mg) and A2 (3,10 mg) with R_f values of 0.48 and 0.44 respectively in methanol were collected. Only fraction A2 showed antimicrobial activity against *Pseudomonas aeruginosa.* From the first column another fraction which was found to be pure was collected as fraction H (68,0 mg) with a R_f value of 0,18 in DCM:EtOAc:MeOH (1:1:2). Compounds A1 and A2 were identified as bulbocapnine (**27**) (spectra 1 & 2) and dicentrine (**21**) (spectra 3 & 4) respectively by comparing their ^{1}H NMR and MS data with those reported in the literature (Guinaudeau *et al.,* 1975). Compound H was identified as crotsparine (**28**) by comparing its MS (spectrum 5), IR (spectrum 6), ^{1}H NMR (spectrum 7) and ^{13}C NMR (spectrum 8 & 9) with those reported in the literature (De Wet *et al.*, 2004; Akendenque *et al.*, 2002).

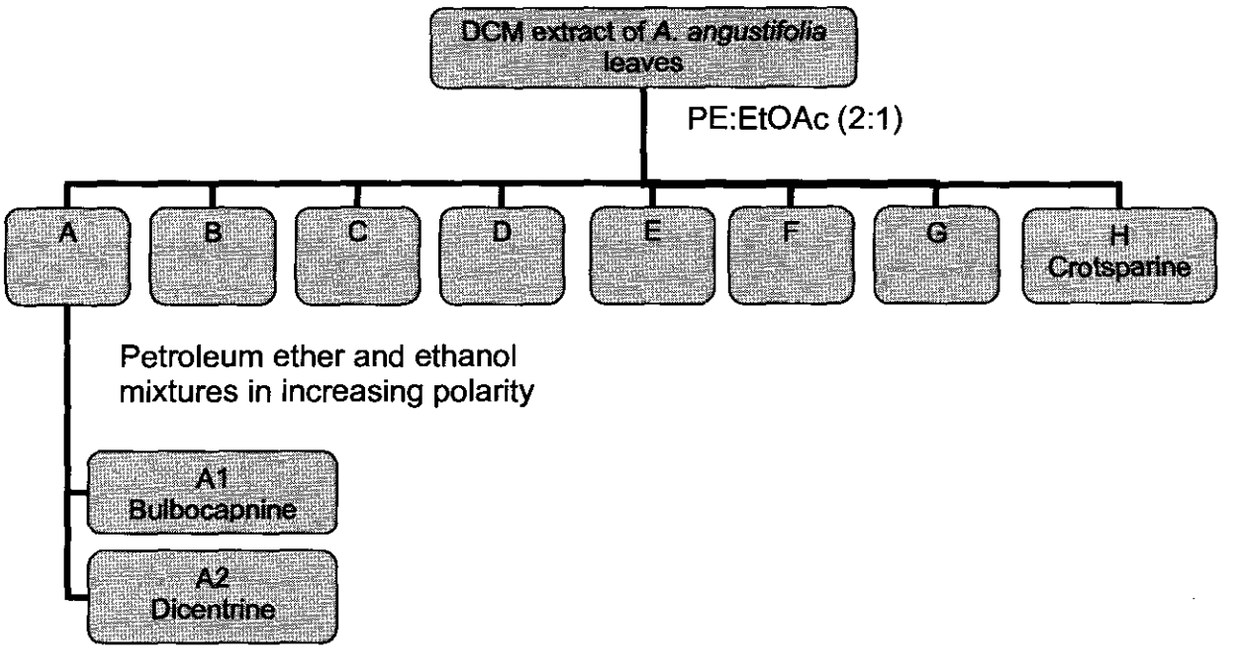


Figure 4.2: Isolation flowchart for the dichloromethane extract *A. angustifolia* leaves.

The ethyl acetate leaf extract (9,107 g) was partitioned between methanol and petroleum ether. Both the methanol and petroleum ether phases were concentrated *in vacuo*. The residue from the methanol phase (8,7 g) was chromatographed on a silica gel column using DCM:EtOAc:MeOH (1:1:2) as a mobile phase. Seven main fractions were collected based on similarities in TLC.

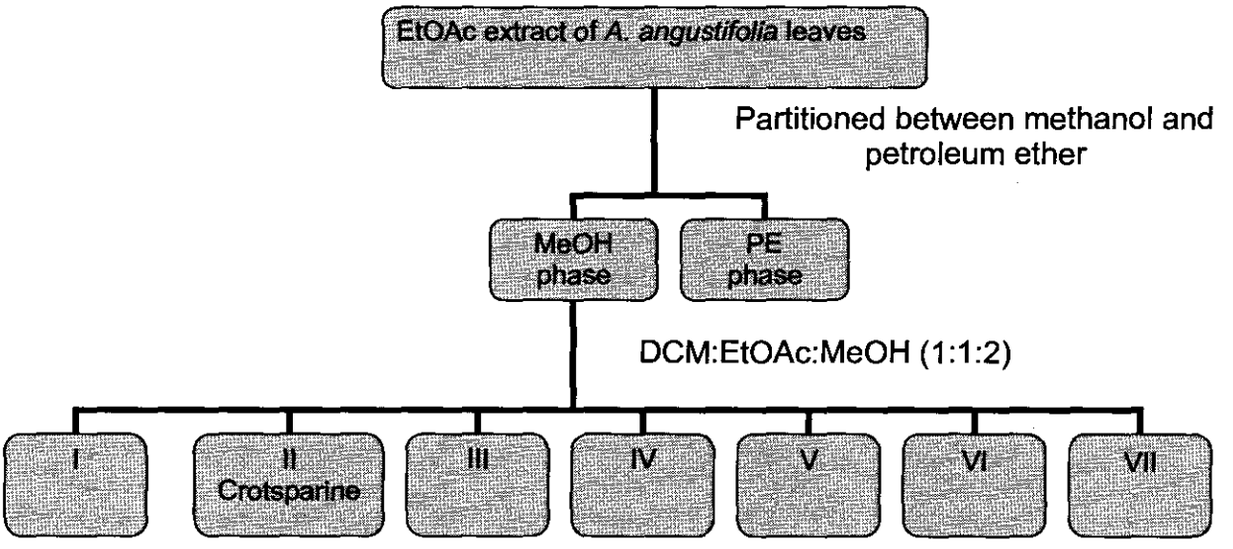


Figure 4.3: Isolation flowchart for the ethyl acetate extract of *A. angustifolia* leaves.

All these fractions were tested for antimicrobial activity, but only fraction II (346 mg), with a R_f value of 0,18 in DCM:EtOAc:MeOH (1:1:2) showed activity against the Gram-

positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*. This fraction was similar to fraction H from the DCM extract, thus identified as crotsparine.

The ethanol extract (17,1 g) of *Antizoma angustifolia* leaves was fractionated on a silica gel column using MeOH:DCM (1:1) as a mobile phase. Nine fractions were collected: Q1, Q2, Q3, Q4, Q5, Q6, Q7, QR and QRR. These fractions were subjected to antimicrobial assay, but only fraction Q3 (5,452 g) with a R_f value of 0,78 in MeOH:DCM (1:1) showed activity against the Gram-positive bacteria *B. subtilis* and *S. aureus*.

Fraction Q3 was further purified by column chromatography with silica gel as a stationary phase and DCM:MeOH (10:1) and DCM:MeOH (5:1) as mobile phases. The collected fractions were tested for antimicrobial activity and only fraction Q3E with a R_f value of 0,18 in MeOH:DCM (1:10) was active against Gram-positive *B. subtilis* and *S. aureus*.

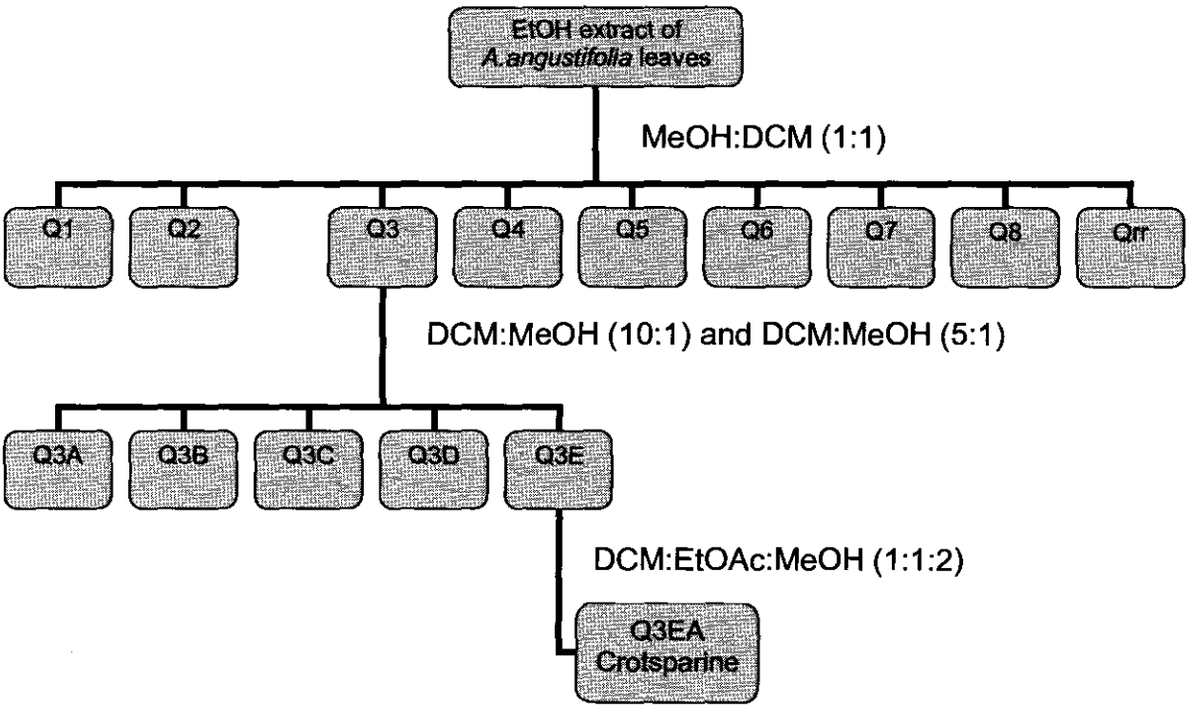


Figure 4.4: Isolation flowchart for the ethanol extract of *A. angustifolia* leaves.

Fraction Q3E was eventually fractionated on a silica gel column once more with DCM:EtOAc:MeOH (1:1:2) as a mobile phase to yield Q3EA (158 mg) with a R_f value of

0,43 in DCM:EtOAc:MeOH (1:1:2). This compound was active against the Gram-positive bacteria *B. subtilis* and *S. aureus*. It was found to be similar to fractions H and II from the DCM and the EtOAc extracts respectively and was thus identified as crotsparine (**28**).

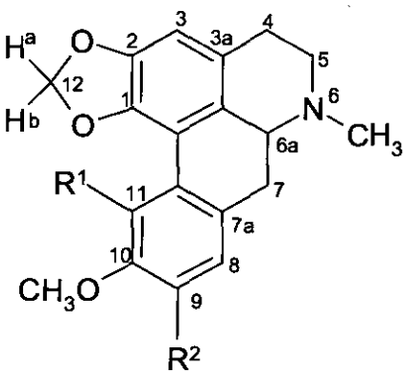
4.6 Characterisation of compounds isolated from *A. capensis* leaf extracts.

4.6.1 Physical data of isolated compounds

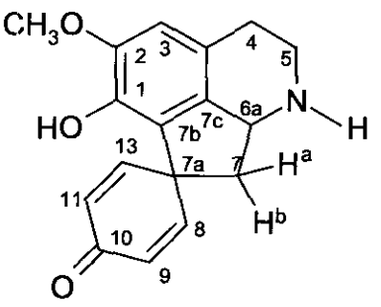
Fraction A1 (bulbocapnine): $C_{19}H_{19}O_4N$; R_f = 0,48; EI MS *m/z* (%), (spectrum 1): 325 M^+ (100); 310 (90); 57 (27); 45 (36); 28 (93); δ_H ($CDCl_3$;spectrum 2); 2,52 (3H, s, N-CH_3); 3,89 (3H, s, OCH_3); 5,93 (1H, d, *J* =1.31, 12a); 6,081 (1H, d, *J* =1,31,H-12b); 6,62 (1H, s, H-3); 6,82 (2H, s, H-8 and H-9).

Fraction A2 (dicentrine): $C_{20}H_{21}O_4N$; R_f = 0,44 (MeOH) ; EI MS *m/z* (%), (spectrum 3): 340 (15); 339 M^+ (75);338 (100); 296 (25), 265 (18);149 (22). δ_H ($CDCl_3$; spectrum 4) 2,53 (3H,s, N-CH_3); 3,90 (6H, s, OCH_3 (9) and OCH_3 (10)); 5,91 (1H,d, *J* =1,44 Hz, 12a); 6,06 (1H,d, *J* =1,44 Hz, H-12b); 6,50 (1H,s, H-3); 6,76 (1H, s, H-8); 7,66 (1H, s, H-11).

Fraction H (crotsparine; dark brown powder): $C_{17}H_{17}NO_3$; mp. (decomposes at 215 °C); R_f = 0,43 (DCM:EtOAc:MeOH 1:1:2); EI MS *m/z* (%), (spectrum 5): 285 (55); 284 (84), 283 M^+ (100); 282 (55); 254 (60); 28 (24); IR (KBr) ν_{max} (spectrum 6; cm^{-1}): 3431,58; 2929,73; 2842,67; 1659,74; 1608,53; 1495,87; 1280,80; 1091,32; 860,88; 466,57; δ_H ($CDCl_3$; spectrum 7) 2,15 (1H, dd, *J* =12,0 and 10,7 Hz, Ha-7); 2,35 (1H, dd, *J* =12,0 and 6,3 Hz, Hb-7); 2,70 (2H, m, H-4); 3,11 (1H, ddd, *J* =12,7; 10,5 and 6,1 Hz, H-5_{ax}); 3,41 (1H, ddd, *J* =12,9; 6.4 and 1,8 Hz, H-5_{eq}); 3,79 (3H, s, OCH_3); 4,24 (1H, dd, *J* =10,6 and 6,4 Hz, H-6a); 6,24 (1H, dd, *J* = 9,9 and 1,8 Hz, H-11); 6,34 (1H, dd, *J* =10 and 1,9 Hz, H-9); 6,54 (1H, s, H-3); 6,82 (1H, dd, *J* = 9,9 and 2,9 Hz, H-12); 6,94 (1H, dd, *J* =10 and 2,8 Hz, H-8); δ_C ($CDCl_3$; spectrum 8 & 9) 25,9 (C-4); 45,1 (C-5); 48,0 (C-7); 50,8 (C-7a); 56,5 (OMe-2); 57,8 (C-6a); 110,4 (C-3); 123,3 (C-7b or 7c); 123,9 (C-7b or 7c); 127,5 (C-11); 128,7 (C-9); 135,6 (C-3a); 141,0 (C-1); 147,4 (C-2); 149,5 (C-12); 153,3 (C-8);186,2 (C-10).



Bulbocapnine (**27**):R^1 = OH, R^2 = H
Dicentrine (**21**): R^1= H, R^2=OCH_3



Crotsparine (**28**)

Figure 4.5: Structures of isolated compounds.

4.7 MIC determination of compounds from *Antizoma angustifolia*

In order to evaluate the antimicrobial activity of the isolated compounds the microplate method was used. The compounds evaluated were crotsparine, dicentrine and bulbocapnine.

First, the concentration of DMSO suitable for use in this assay was determined by the microplate method. The concentration which didn't show an inhibitory effect on the growth of the tested microorganisms (*Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida albicans* and *Staphylococcus aureus*) was 12,5 % (table 4.4).

Table 4.4: MIC values of DMSO against selected microorganimsms.

	B.s	P.a	E.c	C.a	S.a
DMSO (MIC)	50%	25%	25%	25%	50%
Useful conc. for assay	25%	12,5%	12,5%	12,5%	50%

Solutions of crotsparine, bulbocapnine and dicentrine for the assay were prepared. Since 12,5% of DMSO didn't show an inhibitory effect on the range of microorganisms tested, the compounds (table 4.5) were dissolved in 1ml DMSO:H_2O (25%:75%) so that the first set of wells contained only 12,5% of DMSO. However, dicentrine was not properly dissolved in this solvent.

Table 4.5: Compound concentrations in microplate wells.

Compound	Concentration in wells 1-10 (µg/ml)									
Bulbocapnine	1043	521,50	260,75	130,38	65,12	32,59	16,30	8,15	4,07	2,04
Crotsparine	1154	577,75	288,38	144,19	72,09	36,05	18,02	9,01	4,51	2,25
Dicentrine	979	489,50	244,75	122,38	61,19	30,60	15,30	7,65	3,824	1,91

As for the crude extracts, the MIC values of the compounds were determined using the microplate method as outlined in pages 41 to 46. At the prepared concentrations (table 4.5) none of these compounds showed microbial growth inhibition.

The MIC of crotsparine was subsequently determined using higher concentrations (table 4.6.). The remaining amounts of other compounds were insufficient for the preparation of higher concentrations.

Table 4.6: MIC values of crotsparine (µg/ml).

Compound	B.s.	P.a.	E.c.	S.a.	C.a.
Crotsparine	3739	-	1869.5	3739	-

4.8 Summary

Three compounds (i.e. crotsparine, dicentrine and bulbocapnine) were isolated from the plant that showed the best activity, *Antizoma angustifolia*. Leaf extracts were subjected to activity-guided fractionation, using chromatographic techniques. These compounds showed no activity at the concentration of about 1000 µg/ml. However at a higher concentration crotsparine showed some activity against *Bacillus subtilis* (MIC = 3739 µg/ml), *Escherichia coli* (MIC = 1869,5 µg/ml) and *Staphylococcus aureus* (MIC = 3739 µg/ml).

Chapter 5: Discussion and conclusion

5.1 Discussion

The aims of this project were to screen selected South African plants used in traditional medicine for antimicrobia or related activity; to isolate and characterise the compounds responsible for antimicrobial activity from the plant extracts which showed promising antimicrobial activity; to evaluate the antimicrobial activity of the plant extracts and the isolated compounds.

5.1.1. Screening and selection of plants

Eight plants were selected for antimicrobial screening with the aid of the guidelines as described by Baker *et al.* (1995). The selected plants were *Antizoma angustifolia, Carpobrotus acinaciformis, Delosperm herbeum, Melianthus comosus, Physalis viscosa, Rhus pyroides, Zanthoxylum capensis and Ziziphus mucronata*. The procedures used for this screening included the disc diffusion and microplate plate methods.

5.1.1.1 *In vitro* antimicrobial activity of the plant extracts

5.1.1.1.1 Disc diffusion assay

Physalis viscosa root and leaf extracts showed activity against *Pseudomonas aeruginosa* (table 4.1). Its leaf petroleum ether extract also showed activity against *Escherichia coli* though with some intrazonal colonies. *Carpobrotus acinaciformis* twig ethanol extracts and leaf petroleum ether extracts showed inhibition (3 mm) against *Escherichia coli,* also with some intrazonal colonies.

Delosperma herbeum leaf DCM extract showed significant activity (4 mm) against *Escherichia coli* but only slight activity (1 mm) against *Candida albicans. Melianthus comosus* leaf ethanol extract showed marked activity against *Pseudomonas aeruginosa* (4 mm) and *Candida albicans* (7 mm). *Rhus pyroides* leaf extracts showed activity against *Pseudomonas aeruginosa*. *Ziziphus mucronata* extracts had activity against *Bacillus subtilis* (4 mm) and *Candida albicans* (3 mm).

Zanthoxylum capensis extracts showed significant activity against *Candida albicans* only with the ethyl acetate extracts of its twigs showing the best activity (8 mm). More than 50% of the extracts screened were active against *Candida albicans. Aspergillus niger* seems to be resistant to all plant extracts screened in our study.

The *Antizoma angustifolia* extracts showed marked activity against *Baclilus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. This plant showed the widest spectrum of activity amongst the plants screened in this study (table 4.1). For this reason further studies were conducted on *Antizoma angustifolia* extracts with the aim of finding the compound(s) responsible for its antimicrobial activity.

5.1.1.1.2 MIC determination

In the microplate assay, the DCM leaf extract of *Antizoma angustifolia* showed activity against *Staphylococcus aureus* (10,25 mg/ml), *Psuedomonus aeruginosa* (10,25 mg/ml), *Escerichia Coli* (1,28 mg/ml) and *Candida albicans* (1,28 mg/ml) with best the activity against *Bacillus subtilis* (0,32 mg/ml) (table 4.3). The rest of the plant extracts showed variable but weak activity against the range of microorganisms.

5.1.1.1.3 Comparison of MIC and disc diffusion assay results

There is an obvious difference between results obtained with the MIC and disc diffusion assays. For example, the DCM and EtoAc leaf extract of *Physalis viscosa* shows good activity on in the MIC assay, yet no activity was recorded for it in the disc diffusion assay. This difference might be attributed to factors such water solubility and diffusibility of the antimicrobial compound through the agar matrix. This can greatly impact on the observed zone of inhibition, such that a very potent inhibitor may produce a relatively small "halo" simply because it is unable to diffuse adequately through the medium. The inverse might also be true for compounds with low activity, but high water solubility.

5.1.2 Isolation of compounds from *A. angustifolia*

Three alkaloids were isolated from *A. angustifolia*. This was not surprising as *Antizoma angustifolia* belongs to the family Menispermaceae that is known to be rich in alkaloids.

Fractions A1 and A2 were identified as bulbocapnine (**27**) (spectra 1 and 2) and dicentrine (**21**) (spectra 3 and 4) respectively by comparing their MS and ^{1}H NMR data with those reported in the literature (Guinaudeau *et al.*, 1975). Although the isolation of

crotsparine (**28**) and bulbocapnine (**27**) were previously reported from this source (De Wet *et al.*, 2004) that of dicentrine (**21**) was never reported. The antimicrobial activity of crotsparine **(28)** has also not been reported before.

The molecular formula of the first compound (fractions Q3EA and H) was established as $C_{17}H_{17}O_3N$, by the MS $[M]^+$ at *m/z* 283 and ^{13}C NMR data. An IR band at 1659,74 cm^{-1} and a ^{13}C NMR signal at δ 186,37 indicated the presence of an α,β-unsaturated ketone. The ^1H NMR spectrum revealed five aromatic proton signals four of which constituted an AA'BB' system, located at 6,24 (1H, dd, *J* = 9,9 and 1,8 Hz, H-11); 6,34 (1H, dd, *J* =10 and 1,9 Hz, H-9); 6,82 (1H, dd, *J* = 9,9 and 2,9 Hz, H-12) and 6,94 (1H, dd, *J* =10 and 2,8 Hz, H-8). The remaining aromatic proton was a singlet at 6,54 (1H, s, H-3). The ^1H NMR data further disclosed the presence of OCH_3 at 3,79 (3H, s, OCH_3). The NMR of this compound revealed 17 carbon signals, analysing for one methyl, three methylenes, six methine and 7 quaternary carbons using DEPT. This compound was identified as crotsparine (**28**) by comparing its MS (spectrum 5), IR (spectrum 6), ^1H NMR (spectrum 7) and ^{13}C NMR data (spectrum 8) with that reported in the literature (De Wet *et al.*, 2004; Akendenque *et al.*, 2002).

5.1.3 *In vitro* antimicrobial activity of compounds from *Antizoma angustifolia*

The compounds isolated from *Antizoma angustifolia* leaves (bulbocapnine, crotsparine and dicentrine) were evaluated for antimicrobial activity using the microplate method. The microorganisms used in this assay were *Bacillus subtilis* and *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans.* Due to solubility problem of dicentrine in the used solvent (25% DMSO:75% H_2O) the results of dicentrine will not be further discussed.

At concentrations of 1,91 to 1302 µg/ml bulbocapnine and crotsparine showed no antimicrobial activity. However at concentrations of 3739 µg/ml, 1869,5 µg/ml and 3739 µg/ml of crotsparine, activity was observed against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* respectively. No activity against *Pseudomonas aeruginosa* and *Candida albicans* was observed (table 4.6).

In the disc diffusion assay crotsparine also showed activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* (table 4.7) but not against *Psuedomonas aeruginosa* and *Candida albicans*.

5.2 Conclusion

The screened plant extracts showed variable activity against the range of microorganisms. This activity justifies the ethnopharmacological use of these plants in treatment of infections.

The compounds isolated from *A. angustifolia* (bulbocapnine and crotsparine) were not found to be active against *Bacillus subtilis, Staphylococcus aureus, Psuedomonas aeruginosa*, *Escherichia coli,* and *Candida albicans* at the concentration of about 1000 µg/ml. Crotsparine (**28**) exhibited weak antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* with the MIC values of 3739.0, 3739.0 and 1869.5 µg/ml respectively.

From the MIC data, it is clear that crotsparine is not the only compound responsible for the antimicrobial activity of *Antizoma angustifolia* leaf extracts. Additional studies should be conducted to identify further compounds responsible for, or contributing to the antimicrobial activity of this plant. Possible synergisms among its phytochemicals should also be considered.

The activity of crotsparine (**28**) against the microorganisms based on its MIC values is also far lower than its reported antiplasmodial activity. Crotsparine was found to have IC_{50} values of 3,2; 2.1 and 3.4 µg/ml against chloroquine-resistant K1 and FcB1 and chloroquine-sensitive Thai strains of *Plasmodium falciparum*, respectively (Akendenque *et al.*, 2002). Crotonosine, an isomer of crotsparine (**28**) was reported to have biological activities, which included local anaesthetic, neuromuscular-blocking activity and cholinesterase-inhibiting activity (Gaskin & Feng, 1967). Taking this into account, it is important that crotsparine should also be subjected to further biological screening programs.

TLC–bioautography guided isolation is recommended for future studies as it pinpoints the active compound(s) in the crude extract and thus prevent isolation of inactive compounds.

Although the activity of crotsparine (**28**) was not very good, it might still be useful as a lead compound in antimicrobial drug development. The biological activity of these compounds however confirms the fact that the diverse chemistry of plants is still a very important source of novel biologically active and lead compounds.

6 References

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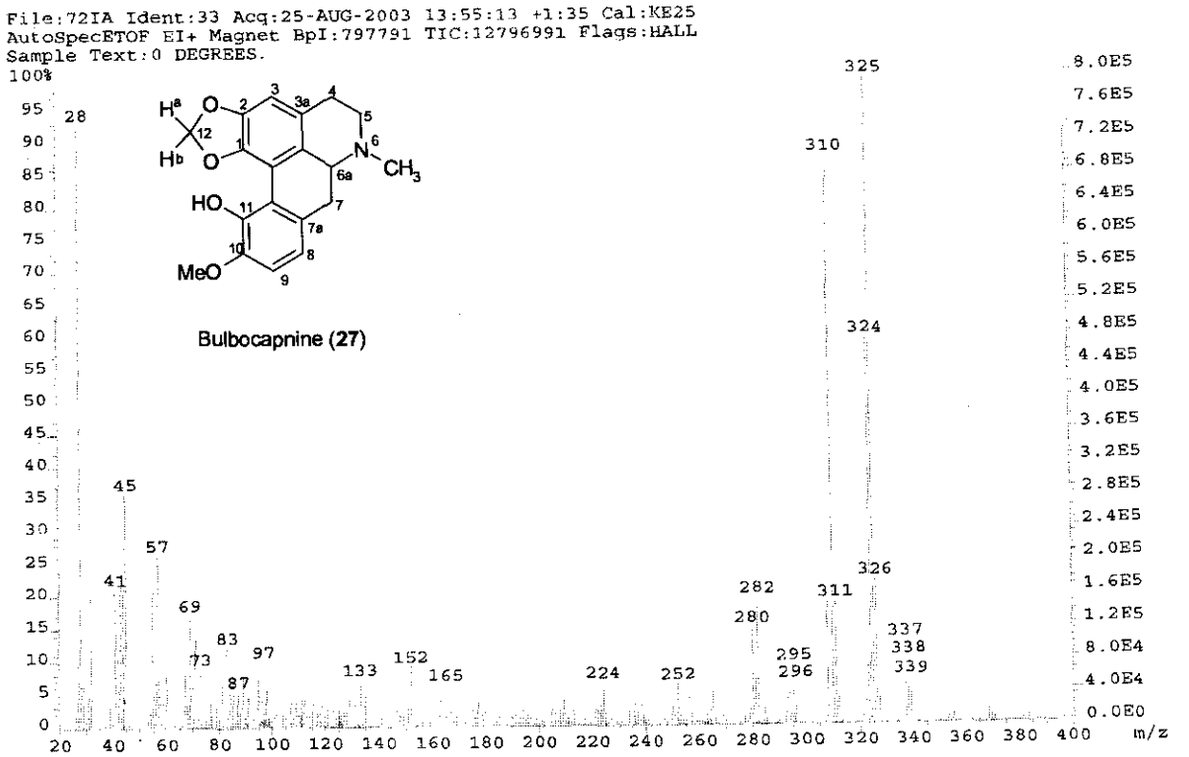

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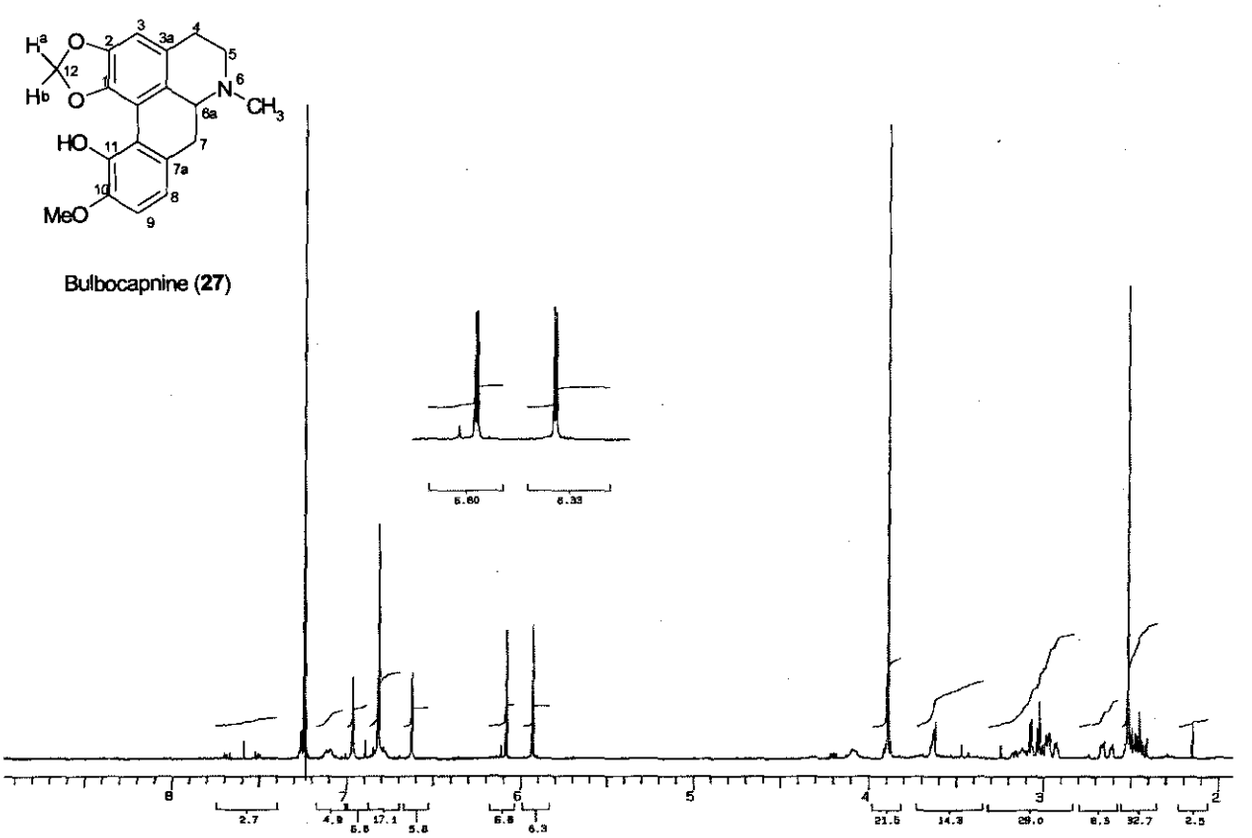

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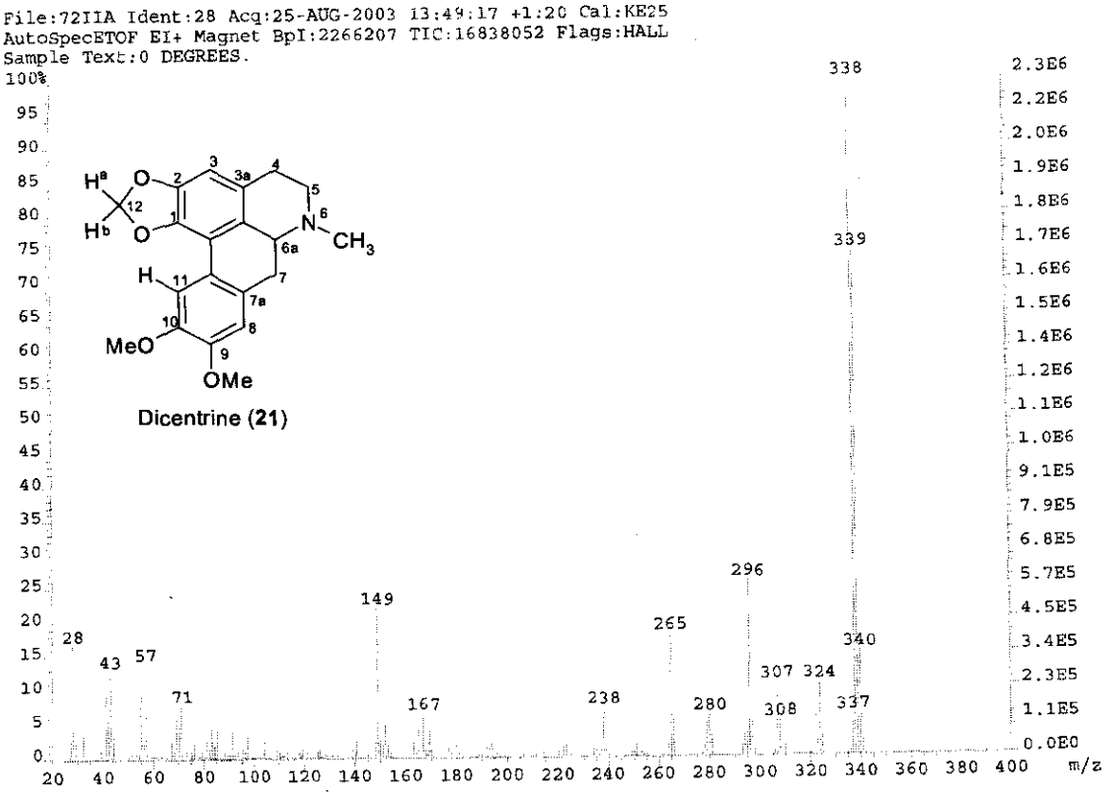

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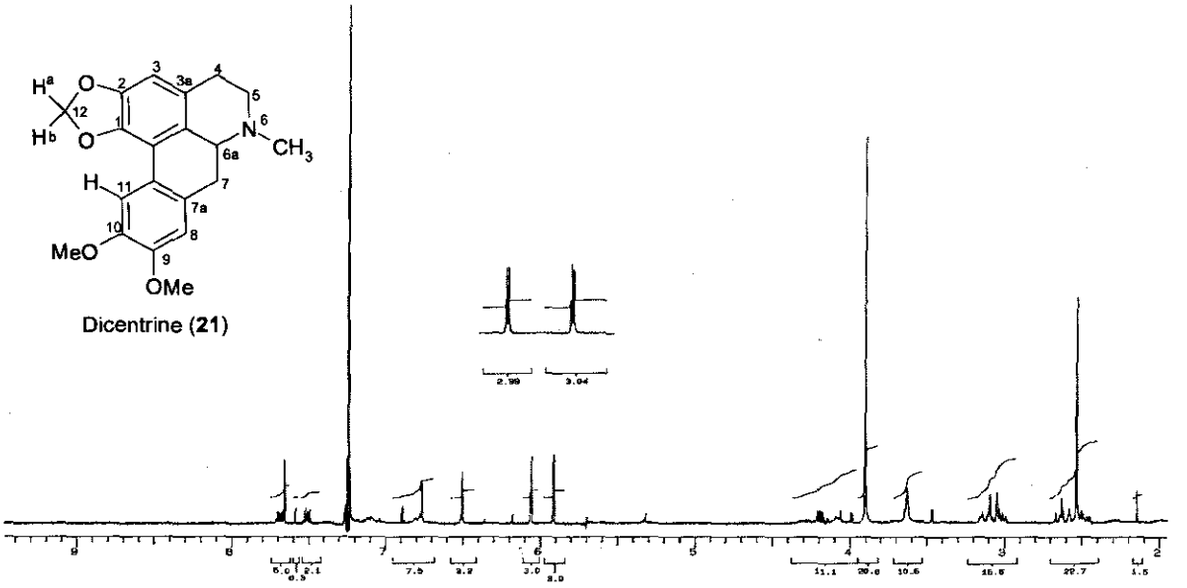

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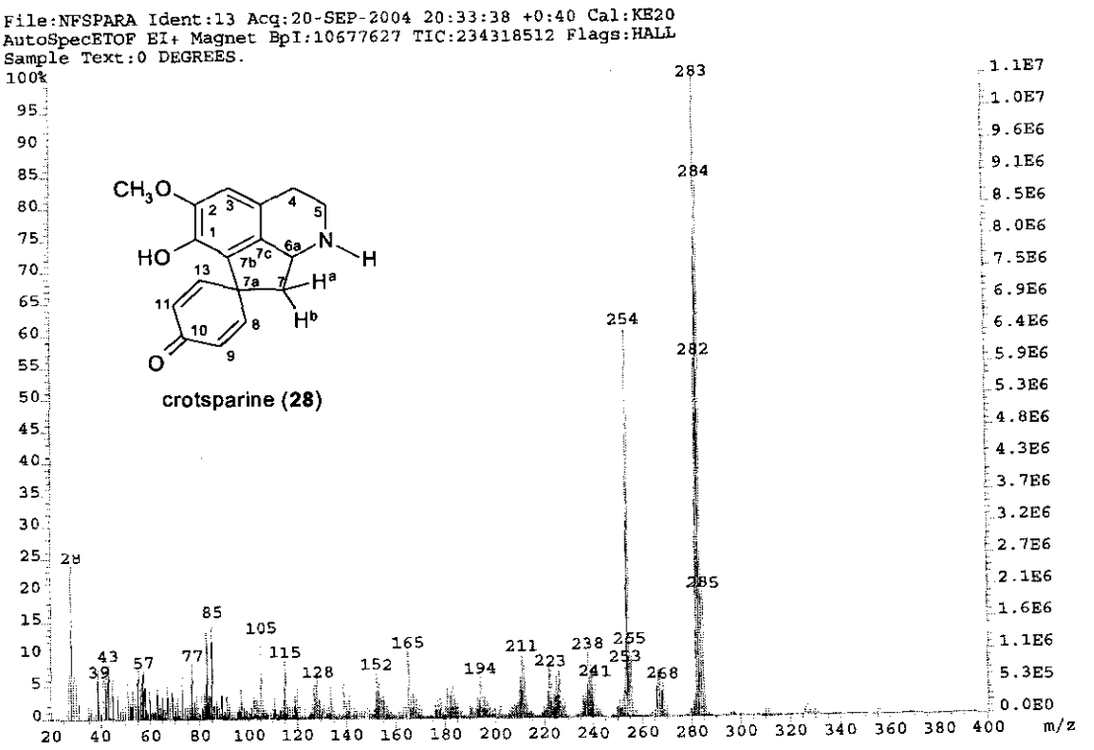

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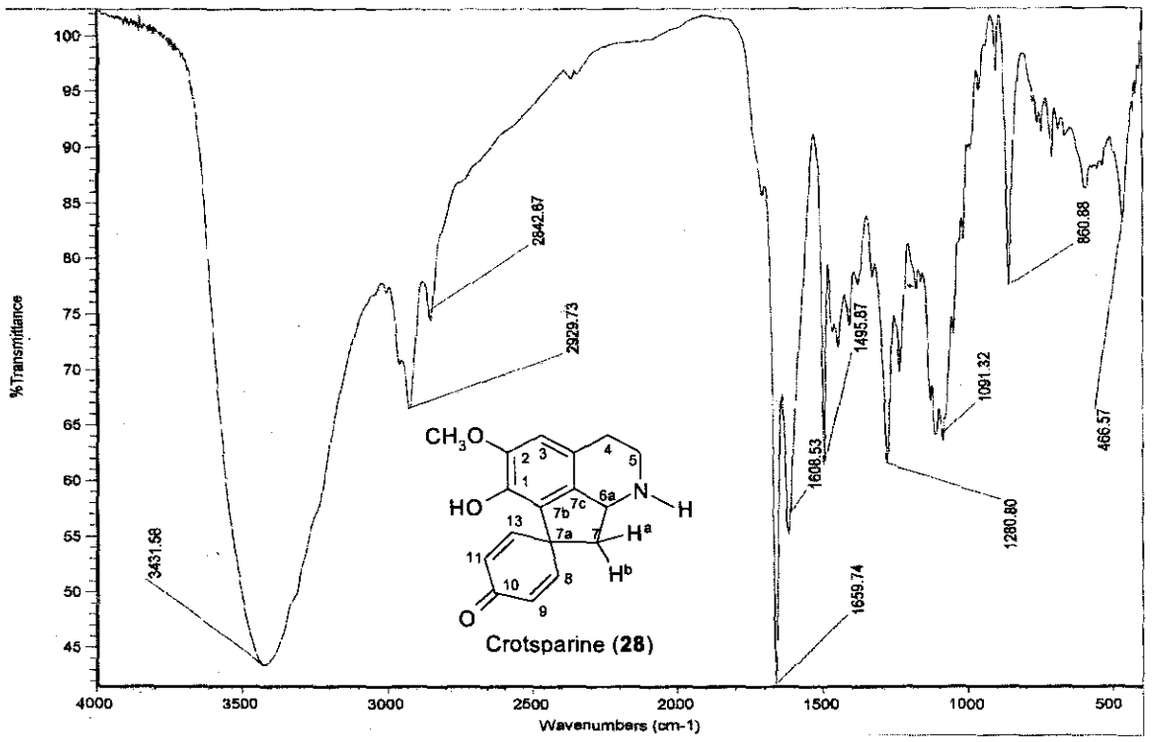

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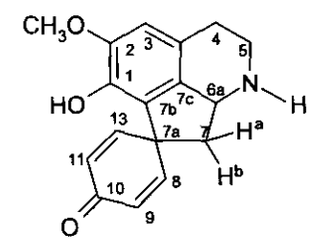

Crotsparine (**28**)

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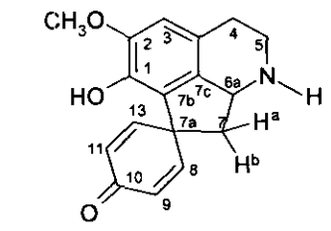

Crotsparine (**28**)

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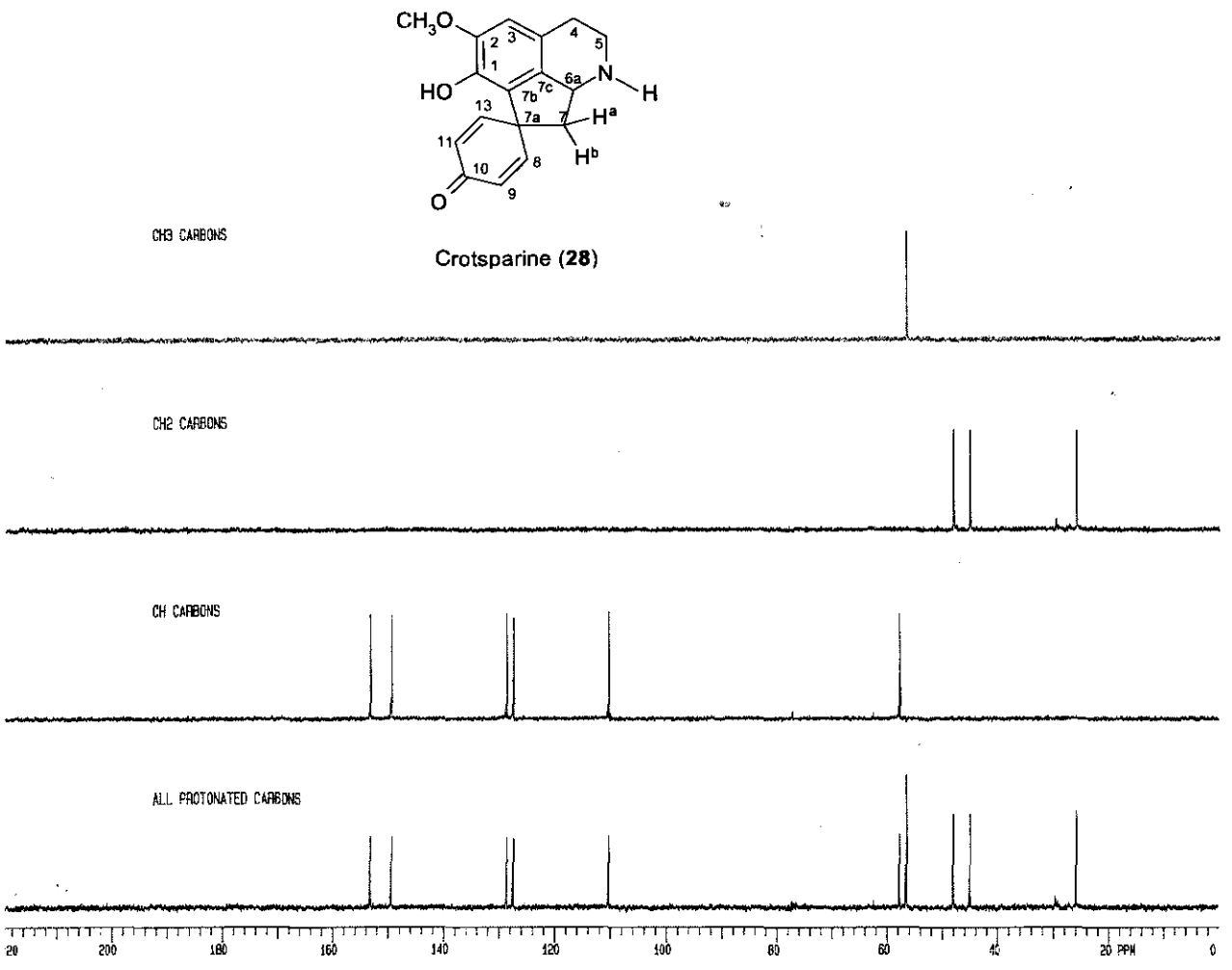

Crotsparine (**28**)