Chapter 1

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Chapter 1

Introduction

1.1 General background and introduction

1.1.1 Occurrence and treatment of *Mycobacterium tuberculosis*

Mycobacteria are believed to be amongst the oldest bacteria on earth. They are free-living organisms to be found in soil, animal dung, water, mud flats and attached to grasses and algae. It has been speculated that cattle were the source of human tuberculosis (TB) infection and that *Mycobacterium tuberculosis* (Fig. 1.1) is a mutant form of *M. bovis* (Evans, 1998).

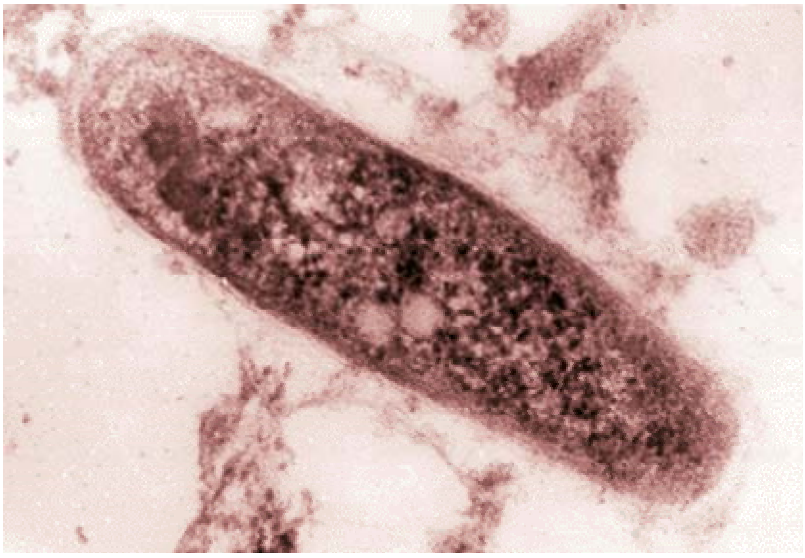


Fig. 1.1: Electron microscope image of *M. tuberculosis* (http://www.abc.net.au/science/news/img/tb.jpg)

According to the Global TB Alliance annual report (2004/2005), over 2 billion people carry the *M. tuberculosis* bacterium. Millions of these infected people die each year. TB also forces people to forgo 12 billion US dollars per annum on treatment and lost income. Most TB patients must complete 130 doses – up to eight tablets a day over a period of 6 months, while multidrug-resistant TB takes 2 years to treat. TB is also the leading killer of people with HIV-Aids, as the current therapy cannot be combined easily with most HIV therapies. The current treatment of TB patients relies on a combination of drugs (Fig.1.2) that must be administered over a period of 6 months.

In 1944 streptomycin was discovered and found to be active (bacteriostatic) against *M. tuberculosis* (Schatz & Waksman, 1944). Due to antibiotic resistance after 2-3 months, the drug had to be taken according to a special rhythm or regime. Soon thereafter, *para*-aminosalicylic acid was discovered to have bacteriostatic activity against TB (Lehman, 1946), and it was found that the combination of the two drugs could be administered without the development of resistance. In 1952 a new drug was discovered, isoniazid, and it was realised that in combination with streptomycin it was the most effective remedy available at the time. With modern drug therapy (including pyrazinamide (found in 1954), ethambutol (1962) and rifampicin (1969)), it was believed that all that was necessary to treat TB, was to take the correct drugs in the correct dosage for the correct duration, for as "short" a period as six months. The problems that developed with the above mentioned treatment regimes, are that the cost and duration of treatment meant that many people were not cured completely. This caused the disease to remain infectious and to become multi drug- resistant (MDR). Due to mutations and the ever-present drug-resistance there is always a need to find new drugs against TB and especially MDR TB, which will be relatively cheap and that will shorten the duration of treatment.

The two naphthoquinones, diospyrin and 7-methyljuglone**,** previously isolated (Lall & Meyer, 2000) in our laboratory did show bactericidal activity against MDR strains of tuberculosis. The results also indicated that the duration of treatment could probably be shorter than treatment with current drugs.

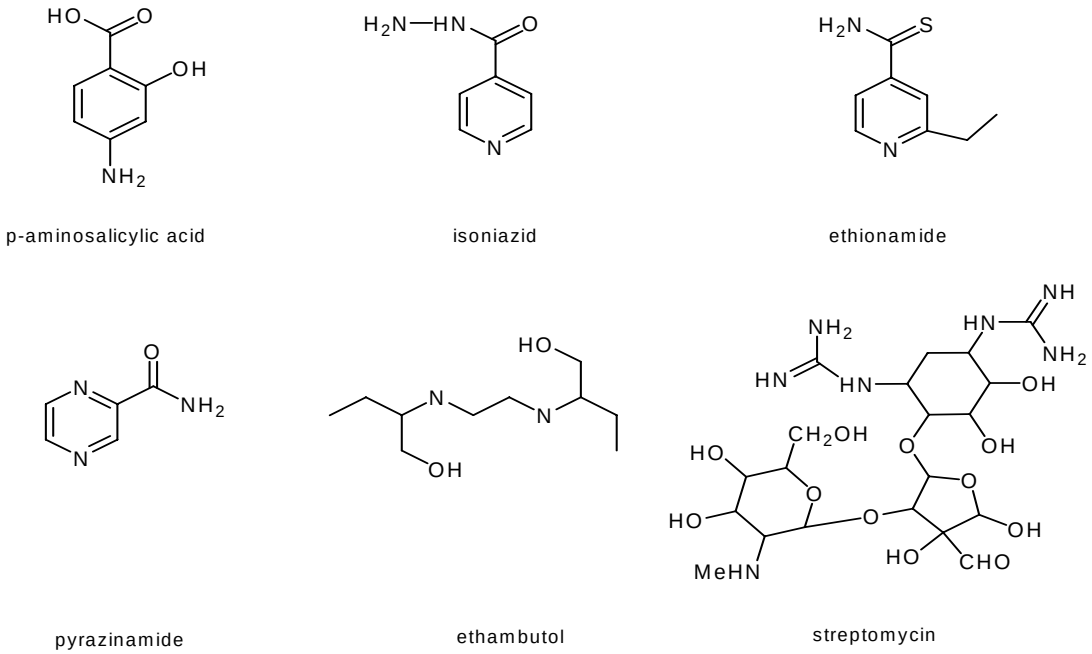


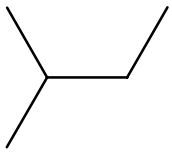
Fig. 1.2: Structures of some antimycobacterial drugs (Young, 1994)

1.1.2 Natural product chemistry

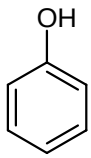
Natural product chemistry or the research into secondary metabolites from higher plants and other organisms has been conducted for centuries. Plants produce a large and diverse array of organic compounds that appear to have no direct function in growth and development (Taiz & Zeiger, 2002). These substances are known as secondary metabolites or natural products. Unlike primary metabolites, such as non-protein amino acids, nucleotides or carbohydrates, secondary metabolites have no generally recognised role in the processes of photosynthesis, respiration, solute transport and other metabolic pathways. Secondary metabolites also differ from primary metabolites in having a restricted distribution in the plant kingdom. A particular secondary metabolite may only be found in a certain plant species or a taxonomically related group of species whereas primary metabolites are found throughout the plant kingdom.

Plants use these secondary metabolites in order to defend themselves against herbivores and pathogenic microbes. In addition to defence, secondary metabolites may also play an important role in other functions, such as structural support (e.g. lignins) or pigmentation (e.g. anthocyanins). There are three classes of important secondary compounds:

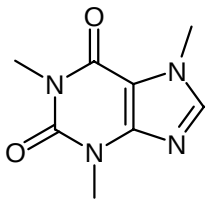
a) Terpenes – consisting of isopentane units (5-carbon elements).



b) Phenolics - containing a hydroxyl functional group on an aromatic ring.



c) Nitrogen-containing compounds – e.g. alkaloids like caffeine, found in coffee.



Caffeine

From these three classes of secondary metabolites, thousands of different compounds have been isolated and characterised. They have many different functions (several have no known function), which relate to the chemical structure of the compound, in the plant.

The compounds that were investigated during this study are part of the phenolic group. The exact biosynthetic pathway of the naphthoquinones has not yet been confirmed and four different biosynthetic pathways for the formation of these compounds have been described (Mallavadhani *et al.*, 1998):

1) Incorporation of shikimic acid into the benzenoid naphthoquinone ring with retention of the carboxyl group.

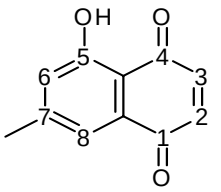
2) Homogentisic acid pathway involving the condensation of mevalonic acid and toluhydroquinone.

3) Prenylation of p-hydroxybenzoic acid with geranyl pyrophosphate followed by decarboxylation and ring closure.

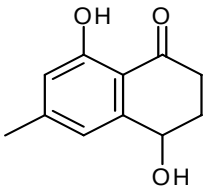
4) The polyacetate-melonate pathway.

According to Chapman & Hall (2006), twelve secondary metabolites have been isolated from *Euclea natalensis.* Nine of these compounds are naphthoquinones. The other three compounds are two dihydroxyursanoic acids (lactone derivatives) and one tetrahydroxyflavanone arabinopyranoside.

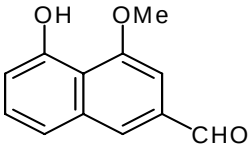
During previous studies two additional compounds have been isolated and characterised from *E. natalensis* for the first time. These compounds, neodiospyrin and 5-hydroxy-4-methoxy-2-napthaldehyde have been isolated previously from other biological sources (Mallavadhani *et al.*, 1998). Fig. 1.3 illustrates the compounds isolated by the author during previous studies (Van der Kooy, 2003). These naphthoquinones has been used during some of the experiments conducted in this thesis.



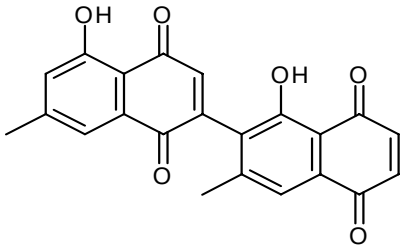
7-Methyljuglone



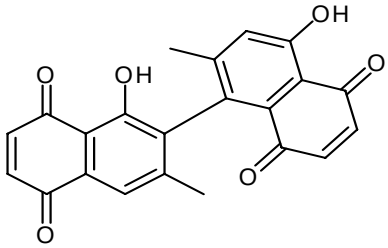
Shinanolone



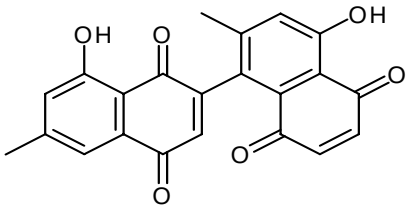
5-hydroxy-4-methoxy-2-naphthaldehyde



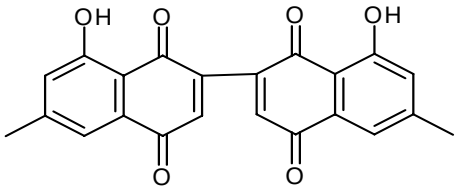
Diospyrin



Isodiospyrin



Neodiospyrin



Mamegakinone

Fig. 1.3: Compounds previously isolated by the author from *Euclea natalensis* (Van der Kooy, 2003). The numbering system is indicated for 7-methyljuglone.

1.1.3 Organic synthesis

Organic chemistry or the study of organic compounds dates back to the mid eighteenth century. In 1770 the chemist Thomas Bergman was the first person to distinguish between organic and inorganic substances. In 1816 the chemist Michel Chevreul found that soap

contained several pure organic compounds, which he termed fatty acids. Friederich Wohler discovered in 1828 that it was possible to convert the inorganic salt ammonium cyanate into the known organic compound urea (McMurry, 1996). Organic synthesis of compounds plays a very important role in biological sciences. When a compound is isolated from a biological source the structure can be determined by spectroscopic techniques. The isolation process itself is often quite difficult and expensive. Furthermore the yields are often low and the environment might suffer from large-scale collection or harvesting of plant material.

The synthetic approach therefore has the following advantages: The target compound can be produced on a large scale. It can be more ecologically friendly in certain cases and it can prove that the proposed isolated structure is correct. It is also in some cases far cheaper to synthesise a compound than to isolate it from its biological source. The first step in the synthesis of a compound is to study the structure of the compound including the functional groups that the carbon skeleton contains as well as the possible isomeric forms (optical, geometric and conformational isomers) that might exist. During this study 7-methyljuglone and three of its dimeric forms have been synthesised.

1.1.4 Stability and solubility of naphthoquinones

The various tests that have to be performed to investigate these compounds as potential TB drugs necessitate the use of various solvents or carriers. This is needed to determine an accurate MIC, which is usually done in buffered solutions or to determine the toxicity in various models, each one often using a different solvent or carrier. It is therefore very important to test these compounds for their solubility and stability in order to get accurate results.

The two terms, solubility and stability, are very closely related in chemical terms. To dissolve a compound one must remember that a chemical reaction is taking place. This reaction takes place between the different functional groups of the compound and the solvent. It is therefore better to call the process solvation instead of dissolving (Morrison & Boyd, 1992). As soon as this reaction is stopped (the solvent evaporated) and the compound remains unchanged then it can be said that the compound was stable in that particular solvent for a specific time at a specific temperature and pressure.

1.1.5 Toxicity of naphthoquinones

Toxicology is the subject concerned with the study of the noxious effects of chemical substances on living systems. The amount of foreign chemicals (xenobiotics) to which humans are exposed has been growing rapidly during the past century. These include drugs, pesticides, environmental pollutants, food additives and industrial chemicals.

The interaction of xenobiotics on the human body is two-fold. There is an effect of the organism on the compound and an effect of the compound on the organism. The first effect includes absorption, distribution, metabolism and excretion (ADME). The effect of the compound on the body can be seen as the mode of action; interaction with proteins and macromolecules, enzymes and receptors and the types of toxic responses produced. The toxicity of any compound relates strongly to the dose of the substance, the type of substance, the frequency of exposure and the type of organism. Toxicity is therefore a relative phenomena and it can be said that there are no harmful substances, only harmful ways of using substances (Timbrell, 1991). To test the toxicity of compounds is therefore quite daunting. For obvious reasons people cannot be used to test the substances initially. Therefore animal tests and various cell lines are available to test the toxicity of compounds. After these tests have been completed it can be tested on people at relevant doses in clinical trials. During this study the toxic effect of the naphthoquinones has been tested on (vero) monkey kidney cells and in mice. In addition the lead compound was also tested on *Musca domestica* (house fly) in an effort to better understand the biological effect in diverse biological systems.

1.1.6 Structure-activity relationship

According to Silverman (2004), Crum-Brown and Fraser suspected in 1868 that the ammonium character of the arrowhead poison, curare, was responsible for its paralytic properties. They tested various ammonium salts and quaternized alkaloids in animals and from this data concluded that the physiological action of a compound was a function of its chemical constitution. These observations were the basis for the study area of structure-activity relationships. Compounds (drugs) can be classified into structurally specific and structurally non-specific drugs. The specific drugs act at a specific site such as a receptor or enzyme. Small changes in their molecular structure have a large influence on their potency. Furthermore molecules with similar biological activities tend to have common structural

features. Non-specific drugs have no specific target and they tend to have lower potency. Similar biological activities might be caused by a variety of structures.

The aim of a structure-activity relationship (SAR) study is therefore to synthesise as many analogs as possible from the lead compound and to test the effect the structure has on the potency. Several structurally related compounds have been tested in this study for potency against TB to determine the active site (pharmacophore) of the lead compound.

1.1.7 Mode of action studies

The effect of the compound on the body can be seen as the mode of action and this includes the interaction with proteins and macromolecules, enzymes and receptors. In 1878 John Langley (Silverman, 2004) who worked on the alkaloids, atropine and pilocarpine, suggested that both these chemicals bind to an unknown substance in the body. This unknown substance was later termed a receptor. The mode of action can therefore be the binding of the drug molecule (or ligand) to its receptor in the body. This receptor in its bound form elicits a physiological or a biological response.

By knowing where the binding site (receptor) of a drug is, the molecule can be improved to increase the potency and decrease the toxicity. This has led to a more targeted design approach of drugs to bind to specific receptors in recent times. The advantage of the targeted approach over the more conventional random approach is that the molecule can be more easily improved without an extensive SAR study. In the long run this saves time and money.

1.2 Objectives of this study

There are two hypotheses that were investigated during this study namely:

- Due to the structure of 7-methyljuglone it is hypothesised that the compound will have problematic stability, solubility and toxicity characteristics.
- It is also hypothesised that due to the structural similarities between 7-methyljuglone and menaquinone (occuring in the mycobacterial electron transport chain system) it might interfere with mycobacterial respiration.

The primary objectives of this study were to investigate the medicinal chemistry of the lead compound, 7-methyljuglone**,** and some related compounds. Secondly, the mode of action in TB was investigated.

The objectives of this study was to:

- Investigate the occurrence of 7-methyljuglone in some ethnobotanically selected plant species.
- Improve the synthesis of 7-methyljuglone and diospyrin**.**
- Determine the stability of selected naphthoquinones.
- Determine the toxicity of selected naphthoquinones in various carriers used for *in vitro* and *in vivo* bioassays.
- Establish a structure-activity relationship.
- Investigate if the mode of action of naphthoquinones is on the mycobacterial electron transport chain.

1.3 Structure of thesis

This thesis mainly deals with the medicinal chemistry of the lead compound, 7-methyljuglone**.** In some chapters other naphthoquinones have been included in the experiments due to their availability and the relative low cost of the experiments. In other cases (Chapter 8 – the *in vivo* mice experiment) only the lead compound and diospyrin have been used due to the high costs involved.

Chapter 1: The introductory chapter contains the general background of *M. tuberculosis* and the general organic and medicinal chemistry aspects related to this thesis.

Chapter 2: This chapter includes all the relevant literature that could be found on the traditional uses of *E. natalensis*. It also includes the phytochemistry and in a broader sense the ecology and occurrence of this species. The biological occurrence of naphthoquinones in plants and animals as well as the biological activity associated with these naphthoquinones are reviewed. Lastly the chemical synthesis and the mode of action of naphthoquinones are reviewed.

Chapter 3: This chapter includes a chemical profiling study into the occurrence of naphthoquinones (NQ's) in ethnobotanically selected plants. Various plant species have been extracted and tested for the occurrence of NQ's. Three analytical tools, TLC, HPLC and NMR, were used to compare the extracts. The species that did contain NQ's were further fingerprinted and the NQ's identified.

Chapter 4: The chemical synthesis of the lead compound and a dimeric form of it is investigated in this chapter. The optimisation of the synthetic pathways is also discussed in this chapter.

Chapter 5: Due to the importance of stability, this chapter deals with the stability of some of the NQ's in the various solvents and buffers used during all the bioassays. The stability in DMSO, BACTEC buffer solution, toxicity buffer (minimum essential medium) and the buffer used for the *in vitro* mice work were tested.

Chapter 6: This chapter describes all the toxicity bioassays that were performed. The toxicity was tested on vero cells, house flies as well as in mice. Only diospyrin and 7-methyljuglone were tested in mice due to the high costs of these experiments.

Chapter 7: To establish a link between specific functional groups in the lead compound and the potency of the compound, a structure- activity relationship was investigated. Some of the NQ's analysed in this chapter have been bought from commercial sources while others were isolated or synthesised.

Chapter 8: This chapter describes the effect that some of the NQ's have on *M. smegmatis*. Due to the difficulty in culturing and maintaining the cultures and the small quantities of cells that can be extracted, only the lead compound and three derivatives have been tested.

Chapter 9: The general discussion and conclusions are presented in this chapter, as well as the major findings of the research. Suggestions for future research are also discussed in this chapter.

1.4 References

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Chapter 2

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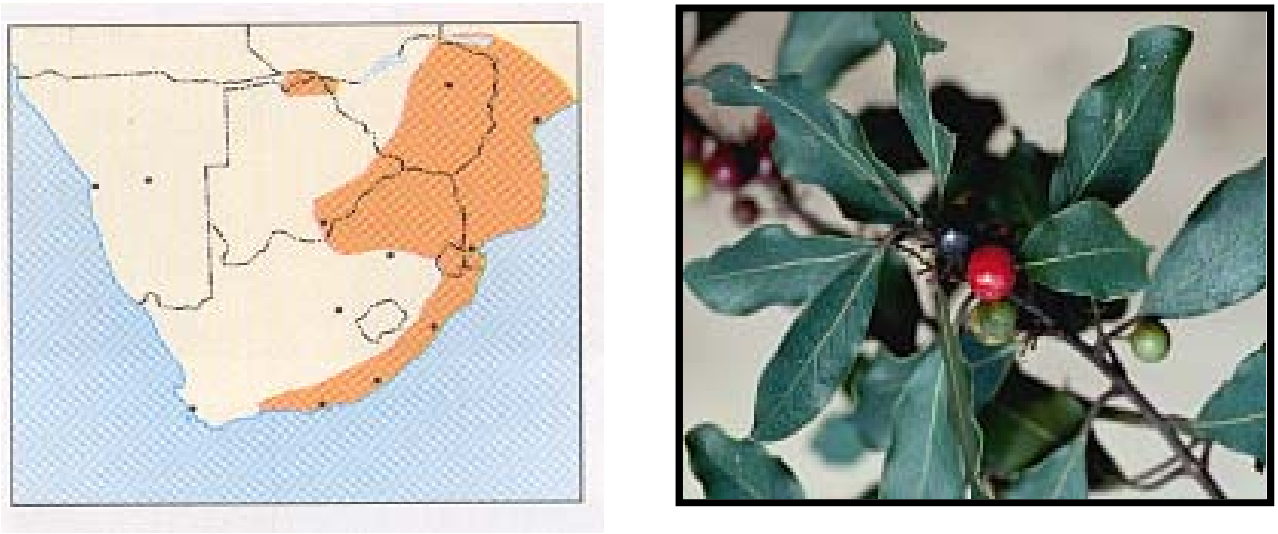
Chapter 2

Literature review

2.1 An introduction to *Euclea natalensis*

The family Ebenaceae consists of about 500 species which is widespread in the tropics and subtropics. In southern Africa two genera are found namely *Diospyros* and *Euclea*. There are sixteen *Euclea* species to be found in southern Africa, with *Euclea natalensis* A.DC. occurring in the Eastern Cape, KwaZulu-Natal and Swaziland (Jordaan, 2003). *E. natalensis* is a shrub or small to medium size tree (Fig. 1.3.a) which grows in coastal and inland forests and also in the bushveld. The leave arrangement of *Euclea* species is very variable and may be opposite to sub-opposite or alternate to whorled even on the same plant. *E. natalensis* has alternate leaves that are elliptic to obovate-oblong, glossy dark green above and densely covered with woolly hairs below. The margins of the leaves appear wavy as shown in Fig. 1.3.b (Van Wyk & Van Wyk, 1997).



(a) **(b)**

Fig. 1.3: Distribution map (a) and leaves and fruit (b) of *E. natalensis* (Van Wyk &Van Wyk, 1997)

2.1.1 Traditional uses

According to Van Wyk & Van Wyk, (1997) the roots of the tree has been traditionally used for dying palm-mats, while decoctions of the roots have numerous medicinal applications as a purgative, analgesic and for its anti-inflammatory properties. The twigs are used as toothbrushes in oral hygiene (Stander & Van Wyk, 1991) and according to Sparg *et al.* (2000) the extracts are used to treat urinary infections and showed good activity against schistosomiasis. The Tonga people use the root for the relief of toothache and headache, while the Zulu people used the roots as a purgative and also for abdominal complaints. The Shangaan people apply the powdered root bark to skin lesions in leprosy and take it internally for ancylotomiasis (Watt & Breyer-Brandwijk, 1962).

2.1.2 Phytochemistry

The amount of research that has been done on this species is relatively small. The publications (24 in total) are mostly on the chemical constituents of *E. natalensis*. Stander and Van Wyk (1991) reported on the use of the root as toothbrushes and speculated that the naphthoquinones in the roots are responsible for the activity against *Streptococcus* species. There are four publications on the antimycobacterial activity of napthoquinones isolated from *E. natalensis* (Lall & Meyer, 1999 & 2001; Lall *et al.*, 2003 & 2005). Weigenand *et al.* (2004) reported on the antibacterial activity of naphthoquinones and triterpenoids from the roots of *E. natalensis*. The compounds isolated from *Euclea* species are given in Table 2.1. In addition two compounds, neodiospyrin and 5-hydroxy-4-methoxy-2-naphthaldehyde, have been isolated recently (Van der Kooy, 2003) from this species.

Table 2.1: Compounds previously isolated from *Euclea* species (Chapman & Hall, 2006)

Compounds	Species
Biramentaceone	*Euclea* spp.
5,8-Dihydroxy-2-methyl-1,4-naphthoquinone	*Euclea* spp.
3,13-Dihydroxy-28-ursanoic acid	*E. natalensis*
3,13-Dihydroxy-11-ursen-28-oic acid	*E. natalensis*
Diosindigo A	*E. natalensis*
Diospyrin	*E. natalensis*
8'-Hydroxy-Diospyrin	*Euclea* spp.
Euclanone	*E. natalensis*
Eucleolatin	*Euclea* spp.
Hydroxyisodiospyrin	*Euclea* spp.
Isodiospyrin	*E. natalensis*
20(29)-Lupen-3-ol	*E. natalensis*
Mamegakinone	*E. natalensis*
7-Methyljuglone	*E. natalensis*
Natalenone	*E. natalensis*
Octahydrodiospyrin	*E. natalensis*
3,4',5,7-Tetrahydroxyflavanone-L-arabinopyranoside	*Euclea* spp.
Xylospyrin	*E. natalensis*

2.2 Occurrence and profiling of 7-methyljuglone in plants

The occurrence of the naphthoquinones studied during this work is widely reported in the Ebenaceae family (Van der Vijver & Gerritsma, 1976; Mallavadhani *et al.*,1998). There are also reports that 7-methyljuglone occurs in some *Drosera* spp. (Caniato *et al*., 1989) and one report that it occurs in thrips where it is used in a defensive secretion (Susuki *et al*., 1995). No other species were reported to contain these naphthoquinones. The structurally similar plumbagin (methyl group on carbon 2) however occurs far more widely in different plant species. Plumbagin occurs in *Plumbago* spp. (Kapadia *et al*., 2005), *Drosera* spp. (Marczak *et al*., 2005), *Diospyros* spp. (Evans *et al*., 1998) and even in the Venus flytrap (*Dionaea muscipula*) (Tokunaga *et al*., 2004). Juglone (lacking the methyl group) occurs predominantly in *Juglans* spp. (Lee *et al.*, 1969). This would give an indication that these structurally similar compounds are produced from different biosynthetic pathways. These molecules are also the parent molecules of a large number of dimers (including diospyrin), trimers and tetramers**.**

During this study ethnobotanically selected plant extracts were profiled in order to determine if there is a link with the presence of naphthoquinones in them. This methodology can be seen as a microscopic metabolomic profiling technique or a targeted metabolomic analysis. Metabolomics is the analytical investigation of an organism's total metabolites in a given extract (Villas-Boas *et al.*, 2005). Plant metabolites can for example be screened for the production of defense compounds when attacked by pathogens, when compared to control plants. It can also be used for quality control purposes for herbal extracts (Yang *et al.*, 2005). Comparisons can also be made between genetically engineered crops and the natural crop. The field of plant metabolomics is quite new. Only 105 articles could be found containing the term "plant metabolomics" when entered as keyword in the CAS database (Scifinder Scholar, 2006). A breakdown of the years of publication indicates that 44 were published in 2005, 29 in 2004, 22 in 2003, 8 in 2002 and only 2 in 2001. No articles could be found before 2001. The search for new medicinal compounds with a metabolomic approach is however a new field and no articles could be found containing this field of study. The analytical techniques usually include sufficient chromatographic separation (HPLC, GC and TLC) with detection carried out by NMR, FT-IR or ESI-MS.

2.3 Chemistry and biological activity of naphthoquinones

2.3.1 Synthesis of naphthoquinones

The synthesis of naphthoquinones and especially 7-methyljuglone and diospyrin has not yet been fully investigated. The first reported synthesis of 7-methyljuglone was done by Cooke & Dowd (1952), using the Friedel-Crafts acylating procedure with the product of step 1 being 8-chloro-7-methyljuglone**.** Musgrave & Skoyles (2001) repeated this procedure with various improvements to the method. The overall yield of the synthesis was still low (approximately 10-20%). Tallman (1984) synthesized 7-methyljuglone with the Diels–Alder reaction during her dissertation. In total there are only 2 published methods for the synthesis of 7-methyljuglone. Only one reference could be found for diospyrin synthesis. Yoshida and Mori (2000) used Suzuki coupling to synthesise diospyrin in a 14-step method, with very low overall yields.

Neodiospyrin was synthesised by Kumari *et al*., (1982) with a redox reaction while Brockmann and Laatsch (1983) successfully synthesised mamegakinone using various

methods. Various synthetic routes are available for the synthesis of plumbagin (Boisvert, 1988), juglone (Khalafy & Bruce, 2002) and menadione via oxidative coupling (Lebrasseur *et al*., 2005). No reports could be found for the synthesis of isodiospyrin and shinanolone**.**

2.3.2 Biological activity of naphthoquinones

The biological activity of the naphthoquinones is given in Table 2.3. The activity of the naphthoquinones is quite diverse which would indicate that the biological activity is species non-specific.

Table 2.3: Biological activity of naphthoquinones with references

Compound	Biological activity	Reference
Diospyrin	Antibacterial	Adeniyi *et al*. (2000)
	Leishmania inhibitor	Hazra *et al*. (2002)
	Tumor inhibitory activity	Hazra *et al*. (2005)
	Anti-inflammatory	Kuke *et al*. (1998)
	Antimycobacterial	Lall *et al*. (2005)
	Antimalarial activity	Likhitwitayawuid *et al*. (1999)
	Topoisomerase inhibitor	Tazi *et al*. (2005)
Isodiospyrin	Antibacterial	Adeniyi *et al*. (2000)
	Termicidal	Carter *et al*. (1978)
	Molluscidal	Gafner & Rodriguez, (1989)
	Antifungal	Ito *et al.* (1995)
	Antimalarial activity	Kapadia *et al*. (2001)
	Anti-inflammatory	Kuke *et al*. (1998)
	Topoisomerase inhibitor	Ting *et al.* (2003)
	Tumor inhibitory	Wube *et al*. (2005)
Mamegakinone	Antimalarial activity	Kapadia *et al.* (2001)
	Leishmaniases activity	Kayser *et al*. (2000)
	Mulloscidal & Fungicidal	Marston *et al*. (1984)
	Tumor inhibitory	Wube *et al.* (2005)
7-methyljuglone	Termicidal	Carter *et al.* (1978)
	Antimicrobial &	

Table 2.3. Continued from page 28.

	cytotoxic Antimycobacterial Ca-channel blocking Antifungal Active against ants Anti-feedant activity Tumor inhibitory Activity	Gu *et al*. (2004) Lall *et al*. (2005) Neuhaus-Carlisle *et al*. (1997) Steffen & Peschel, (1975) Suzuki *et al.* (1995) Tokunaga *et al.* (2004) Wube *et al.* (2005)
Neodiospyrin	Antimycobacterial Tumor inhibitory	Van der Kooy *et al*. (2006) Wube *et al*. (2005)
Shinanolone	Antibacterial & antimycobacterial Anti-tumor	Weigenand *et al*. (2004) Wube *et al.* (2005)

Due to the large amount of publications on juglone**,** menadione and plumbagin these three compounds were not included in the table. The activity of these compounds includes growth inhibition (juglone) (Bohm *et al.*, 2006), antifungal activity (juglone) (Tomaszkiewicz-Potepa & Vogt, 2004), antitumor activity (menadione) (Verrax *et al.*, 2005), antibacterial activity (menadione) (Park *et al.*, 2006), antimycobacterial (plumbagin) (Tran *et al.*, 2004) among others.

Tokunaga *et al.* (2004) showed that naphthoquinones (including 7-methyljuglone) has strong anti-feedant properties. The naphthoquinones are accumulated by carnivorous plants as defence mechanism against predators. 7-methyljuglone also inhibits the protein kinase C which gives the compound antitumor properties (Timothy *et al.*, 1995). Ragazzi *et al.* (1994) tested the compound on pig and precontracted rabbit trachea to assess their pharmacological activity as therapy for respiratory diseases. They found that the high activity and cardiac actions suggests that these compounds should be proposed as drugs for respiratory diseases. 7-Methyljuglone also shows strong termicidal activity (Carter, 1978). Tikkanen (1983), found that the compound has mutagenic activity in the salmonella/microdsome test. Diospyrin shows inhibitory activity of murine tumors *in vivo* and in human cancer cell lines (Hazra, 2005). Diospyrin also indicated some termicidal activity (Ganapaty, 2004).

2.3.3 Mode of action of naphthoquinones

The mechanism of action of naphthoquinones have not yet been fully investigated. The references listed in Table 2.4 refer to the possible mode of action and do not give specific binding or receptor sites. There are reports that the naphthoquinones might have a novel mode of action, which are not yet fully understood (Cushion *et al.,* 2000).

Table 2.4: The mode of action of naphthoquinones and the author references.

Compound	Mode of Action	Reference
Diospyrin	Prevent or reverse topoisomerase I and DNA complex from forming Binds electron transport chain	Bailly (2000) Cushion *et al*. (2000)
Isodiospyrin	Binds topoisomerase I - preventing it from binding to DNA	Ting *et al.* (2003)
Juglone	Inhibited respiration in bean and lettuce plants and binds to thiol groups of peptides	Li *et al*. (1993)
Plumbagin	Superoxide generator	Wang *et al.* (1998)

The mode of action of these compounds remains largely unknown. The publications referred to mainly describe the possible mode of action in uncertain terms. The well studied compounds plumbagin, menadione and juglone**,** which were not isolated from *Euclea natalensis* but commercially obtained, did have various proposed possible mechanisms of action. The articles predominantly refer to the generation of oxygen radical species, which damages cells of various organisms. They were therefore often tested for their possible anticancer properties (Wang *et al.,* 2003).

2.4 References

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Chapter 3

The occurrence and profiling of naphthoquinones in ethnobotanically selected plants

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Chapter 3

The occurrence and profiling of naphthoquinones in ethnobotanically selected plants

3.1 Introduction

The occurrence of the naphthoquinones studied during this thesis is widely reported in the Ebenaceae family (Van der Vijver & Gerritsma, 1976; Mallavadhani *et al*., 1998). There are also reports that 7-methyljuglone occurs in some *Drosera spp*. (Caniato *et al*., 1989) and one report that it occurs in thrips who use it in a defensive secretion (Susuki *et al*., 1995). No other species were reported in containing this naphthoquinone.

According to Van Wyk *et al.* (2002) there are many indigenous plants that are used for coughs, bronchitis and asthma (chest related ailments). It is possible that there's a link between chest problems (TB-like symptoms) and 7-methyljuglone or related naphthoquinones occuring in plants used to treat these symptoms. Nine plant species were selected at random from plants reported to have these properties (Van Wyk *et al.*, 2002). The selected species and the plant parts used traditionally were collected and extracted. The number of compounds and naphthoquinones that has been characterised from each species according to the Dictionary of Natural Products (Chapman & Hall, 2006) is given in Table 3.1.

Table 3.1: The number of compounds isolated from the selected species used to treat TB-like symptoms as well as the number of quinones and naphthoquinones.

Plant species	Family	Compounds isolated	Quinones or naphthoquinones
Dombeya rotundifolia	Sterculiaceae	0	0
Drosera capensis	Droseraceae	11	1
Ekebergia capensis	Meliaceae	23	0
Foeniculum vulgare	Apiaceae	97	0
Leonotus leonurus	Lamiaceae	21	0
Mentha longifolia	Lamiaceae	129	0
Prunus africana	Rosaceae	188	0
Rapaneae melanophloeos	Myrsinaceae	12	3
Ziziphus mucronata	Rhamnaceae	3	0

There are no reports of 7-methyljuglone or related naphthoquinones occurring in any of the above species, excluding *Drosera capensis*. There are reports that some *Drosera* spp. contain 7-methyljuglone while other *Drosera* spp. contain plumbagin**.**

The aim of this chapter is threefold:

- Firstly to establish a possible link between naphthoquinones (especially 7-methyljuglone) and plants used traditionally for treatment of chest ailments. Therefore plants were chosen at random without having any chemotaxonomic ralation to each other.
- Secondly, to establish if specific (groups of) compounds e.g. flavonoids, coumarins etc. are responsible for these plants being used as medicine through a small-scale metabolite profiling experiment.
- Thirdly, if naphthoquinones are present, to identify and quantify them.

The methodology that was employed is small-scale metabolite fingerprinting. Metabolomics (or metabonomics) is a new field of study in science and the exact meaning is not always clear. According to Villas-Boas *et al. (*2005), Stephan Oliver used the word metabolome in 1998 to designate the set of all low-molecular weight compounds that are synthesised by an organism. Soon afterwards Oliver Fiehn published a detailed review on metabolome analysis and introduced the word metabolomics, to designate a comprehensive analysis in which all the metabolites of an organism is identified and quantified. An appropriate definition of metabolomics is probably the following: The characterisition of metabolic phenotypes (metabolome) under specific sets of conditions and the linking of these phenotypes to their corresponding genotypes (Villas-Boas *et al*., 2005).

Metabolomics can be viewed in two different ways. Firstly the microscopic view, which looks at specific groups of compounds (e.g. flavonoids). Secondly the macroscopic view, looks at all metabolites and is therefore the true metabolomics. Metabolite profiling in essence means that the metabolomic extracts are fingerprinted with analytical tools and any correlation between the species would give a positive result. Variation in the concentrations of a compound is also an important factor.

During this study three analytical tools (HPLC, NMR and TLC) were used in order to identify the compound(s) possibly active against a specific disease in a small-scale metabolite

fingerprint. The results should indicate that a specific group of compounds or even a single compound is active against the pathogens related to chest ailments. In order to achieve this various chromatographic and spectroscopic tools need to be used. TLC would be the cheapest, but would not give any structural information. HPLC would give valuable information on the properties of the compounds especially the UV spectrum (with the PDA detector). The most powerful tool is the NMR, which will give structural information. All three of these methods were employed during this chapter.

3.2 Materials and methods

3.2.1 Plant material

The plant species chosen for this study were selected from Van Wyk *et al*. (2002) and are used for coughs, chest pains and other resperitory diseases. The plants were selected so as to contain trees and shrubs. The plant material was collected in the Botanical Gardens of the Universty of Pretoria. Table 3.2 indicates which plants and plant parts were used.

3.2.2 Preparation of extracts

The plant material (50 g) was dried and ground into a fine powder, after which it was quantitatively extracted twice with dichloromethane. The crude extracts were left to dry after which it was subdivided into three fractions for the different analytical analysis.

Table 3.2. Plant species collected with their growth type and parts used traditionally

Plant species	Growth type	Plant part used
Dombeya rotundifolia	Shrub/Tree	Bark
Drosera capensis	Shrub	Above ground
Ekebergia capensis	Tree	Bark, leaves
Foeniculum vulgare	Shrub	Above ground
Leonotus leonurus	Shrub	Above ground
Mentha longifolia	Shrub	Above ground
Prunus africana	Tree	Bark
Rapaneae melanophloeos	Tree	Bark
Ziziphus mucronata	Tree	Bark

3.2.3 Profiling with TLC

Normal phase silica TLC plates (Merck) were prepared and 100 µl of an 1 mg/ml was spotted on the plates. The plates were developed with three different solvent systems.

- Apolar system: Hexane 100 %
- Semi-polar system : Hexane:Ethyl acetate 5:2
- Polar system: Ethanol 75 %: HCl 0.5 %

The plates were developed in duplicate. One plate was analysed by subjecting it to UV while the other plate was dipped into a vanillin:sulphuric acid mixture (7.5 g vanillin:5 ml H_2SO_4 in 250 ml ethanol), after which it was dried and analysed.

3.2.4 Profiling with HPLC

For the identification of naphthoquinones in the samples a 1 mg/ml solution was prepared in acetonitrile and 10 µl injected into the HPLC. Each sample was injected three times. The HPLC consisted of a PDA UV detector set to 254, 325 and 430 nm. A 150mm X 4.6 mm RP 18 silica column was used. The mobile phase was 62 % acetonitrile acidified with 5 % acetic acid. Authentic naphthoquinones were used as standards. For the metabolomic fingerprint the mobile phase consisted of a gradient system of 100 % acidified water changing to 100 % acetonitrile after one hour.

3.2.5 Profiling with NMR

Thirty mg of the crude extracts were dissolved in 0.7 ml of d-chloroform. The samples were dissolved and sonicated, after which it was filtrated into the NMR tube. The ^{1}H-NMR was acquired with 2000 repetitions for each sample. The NMR parameters was set to the following: pw90 = 9.4 µs, sw = 4000 Hz, nt = 2000, delay time = 10 s. After the acquisition was completed the spectra were phased and referenced to chloroform at 7.24 ppm. The vertical scale of the chloroform peak was set to 3000.

The spectra were subdivided into the following three regions and manually compared:

1: aliphatic and allylic region: 0-2.50 ppm

2: halogen and vinylic region: 2.51-6.50 ppm

3: extended aromatic region: 6.51-12.0 ppm

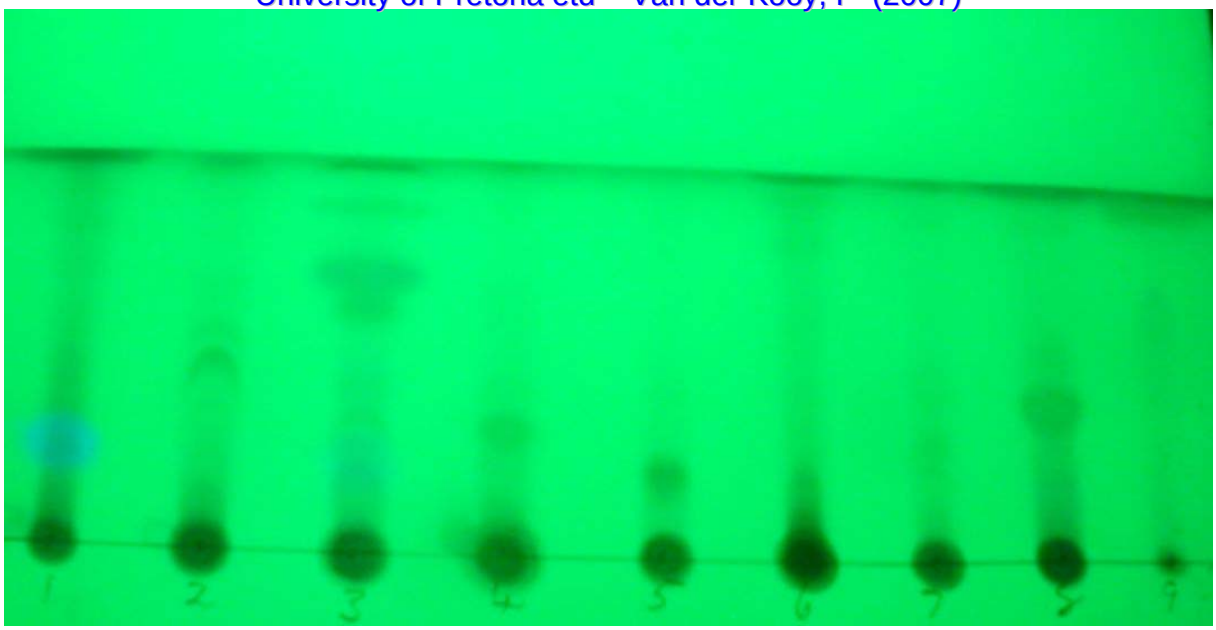
3.2.6 Fingerprinting *Drosera capensis*

The occurrence of naphthoquinones in *Drosera capensis* prompted the further investigation and identification of the compounds in this species. The plants were separated into the flowers, flower stems, leaf lamina, leaf petioles and the roots. The samples were extracted quantitatively with chloroform and subjected to HPLC. The amount of **(10)** in the different plant parts were established from a standard curve prepared from an authentic **(10)** sample. Other naphthoquinones appearing in trace amounts were qualitatively identified with NMR and HPLC.

3.3 Results

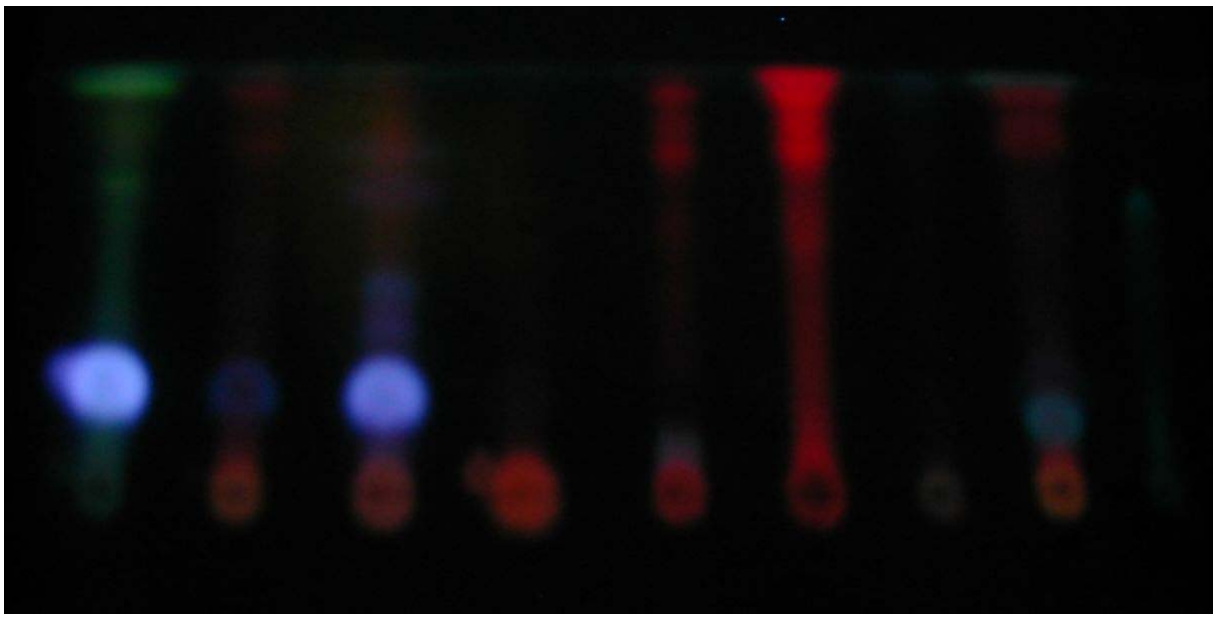
3.3.1 Profiling with TLC

Fig 3.1 and 3.2 illustrate the plates that were developed in hexane:ethyl acetate 5:2 under UV light (at 254 and 365 nm). There are indeed some correlations between the extracts. Samples 1 and 3 (*D. rotundifolia* and *F. vulgare*) appeared to have a coumarin type compound present. This compound was also present in small amounts in the *E. capensis* and *Z. mucronata* sample. From the UV properties and polarity it appears to be a coumarin.



1 2 3 4 5 6 7 8 9

Fig. 3.1. TLC plate of the nine samples under short wave length (254nm) developed in hexane:ethyl acetate (5:2). Lane 1: *Dombeya rotundifolia,* **2:** *Ekebergia capensis,* **3:** *Foeniculum vulgare,* **4:** *Leonotus leonorus,* **5:** *Mentha longiflora,* **6:** *Prunus africana,* **7:** *Rapaneae melanophloes* , **8:** *Ziziphus mucronata* and **9:** *Drosera capensis.*



1 2 3 4 5 6 7 8 9

Fig. 3.2. TLC plate of the nine samples under long wave length (350nm) developed in hexane:ethyl acetate (5:2). Lane 1: *Dombeya rotundifolia,* **2:** *Ekebergia capensis,* **3:** *Foeniculum vulgare,* **4:** *Leonotus leonorus,* **5:** *Mentha longiflora,* **6:** *Prunus africana,* **7:** *Rapaneae melanophloes* , **8:** *Ziziphus mucronata* and **9:** *Drosera capensis*

3.3.2 Profiling with HPLC

There was no apparent overlap of any compounds in the samples. The only plant that did contain 7-methyljuglone (Fig. 3.3) as well as the dimeric naphthoquinones: mamegakinone and neodiospyrin was *D. capensis*. No reports in literature could be found reporting the dimeric compounds in *Drosera* species. The major naphthoquinone was 7-methyljuglone. All of these naphthoquinone's identities were confirmed with proton NMR.

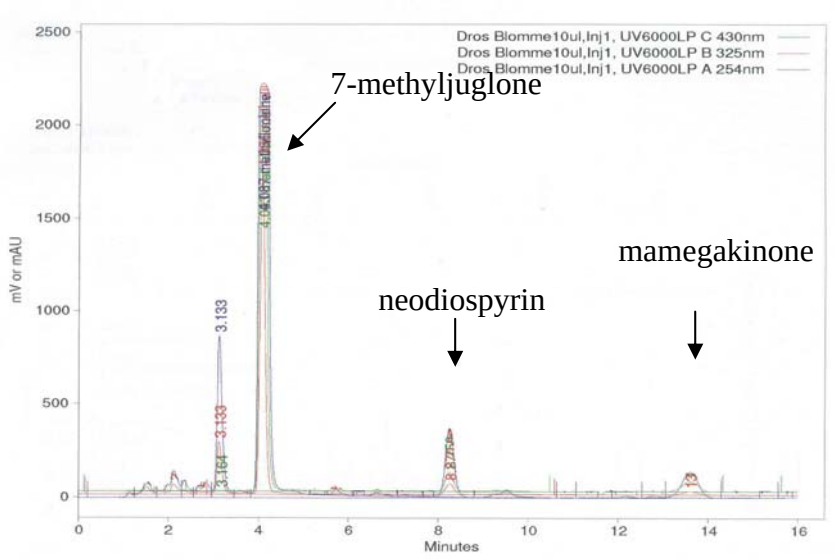


Fig. 3.3. HPLC chromatogram indicating the presence of 7-methyljuglone, neodiospyrin and mamegakinone in the *D. capensis* crude extract.

The mobile phase that was employed was specifically developed for the detection of 7-methyljuglone and its dimeric forms. Due to the absence of a degasser, the gradient mobile phase for the fingerprinting did not give adequate results. It was therefore not further investigated. The ideal fingerprint on a HPLC should employ a gradient system starting with water and ending after an hour with 100 % acetonitrile.

3.3.3 Profiling with NMR

The subdivided spectra were compared with each other. Due to the complexity of region 1 only regions 2 and 3 were compared. Fig 3.4-3.12 show the NMR spectra of all the samples. The NMR confirmed the presence of 7-methyljuglone**,** neodiospyrin and mamegakinone in *D. capensis*. It also confirmed the absence of these compounds in the rest of the samples. The

main region of interest was the aromatic region and the region between 9-10 ppm which is expanded in the figures (excluding *D. capensis*). All the samples contained similar peaks indicating that certain compounds are present in most of the extracts.

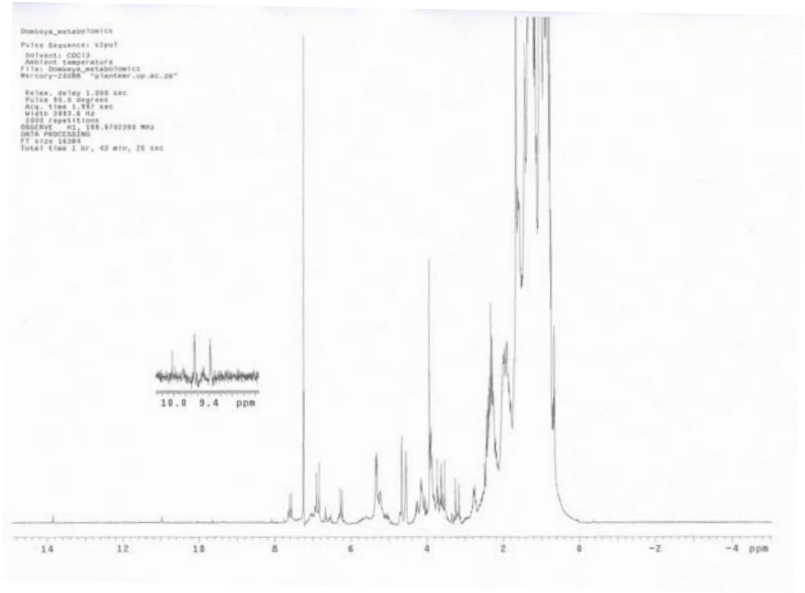


Fig. 3.4. The ^{1}H -NMR spectrum of *Dombeya rotundifolia*. The region between 9-10ppm is indicated in the inset.

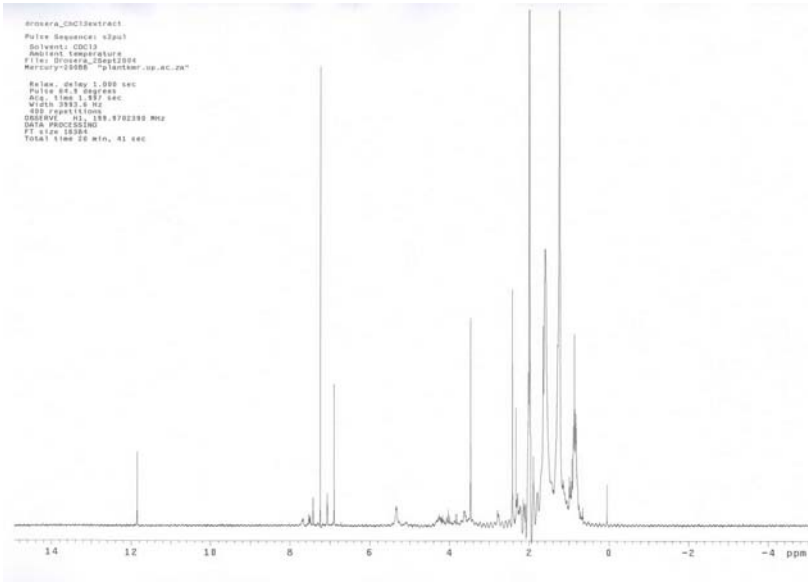


Fig. 3.5. The ^{1}H -NMR spectrum of *Drosera capensis.*

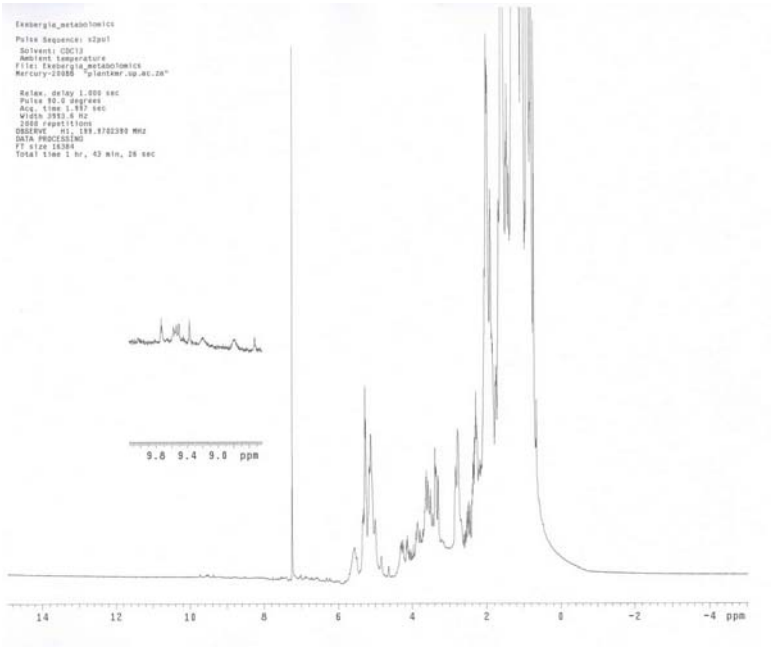


Fig. 3.6. ^{1}H -NMR spectrum of *Ekebergia capensis.*

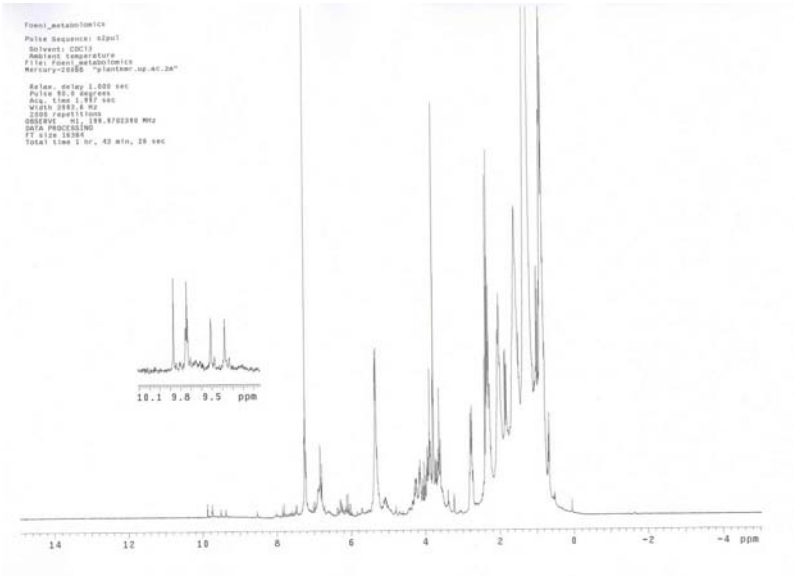


Fig. 3.7. ^{1}H -NMR spectrum of *Foeniculum vulgare.*

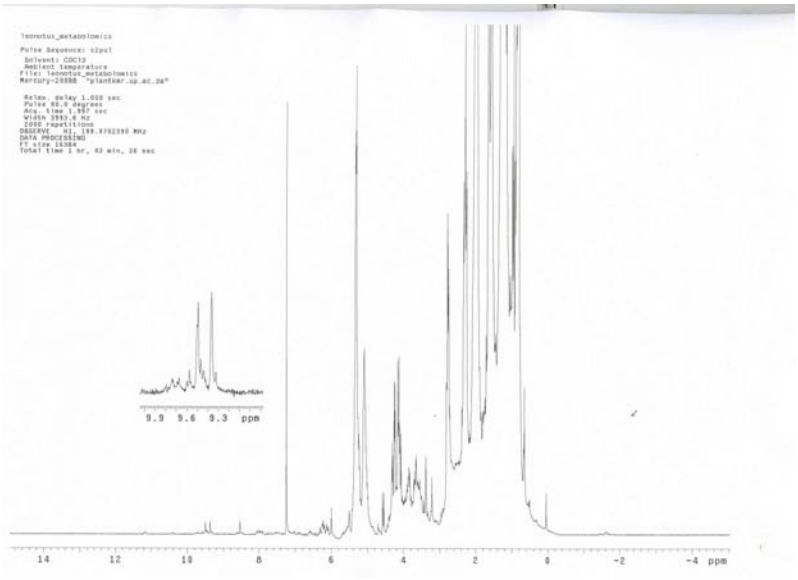


Fig. 3.8. ^{1}H -NMR spectrum of *Leonotis leonorus.*

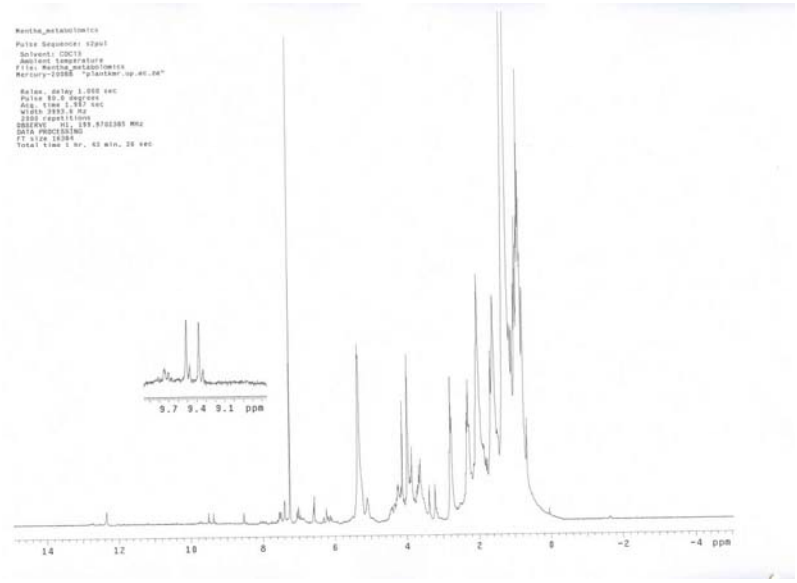


Fig. 3.9. ^{1}H -NMR spectrum of *Mentha longifolia.*

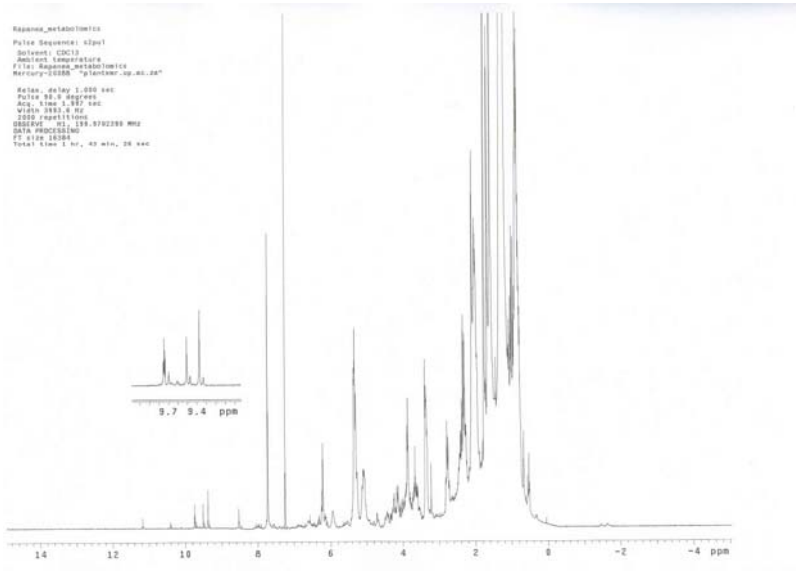


Fig. 3.10. ^{1}H -NMR spectrum of *Rapanea melanophloes.*

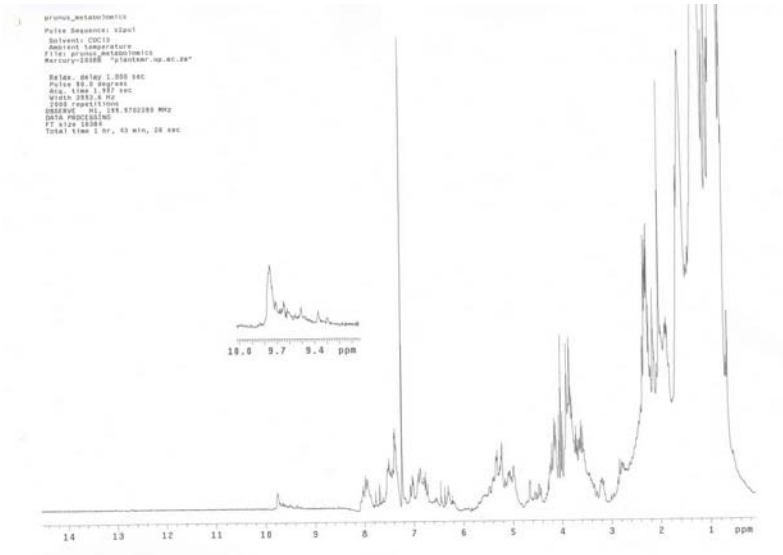


Fig. 3.11. ^{1}H -NMR spectrum of *Prunus africana.*

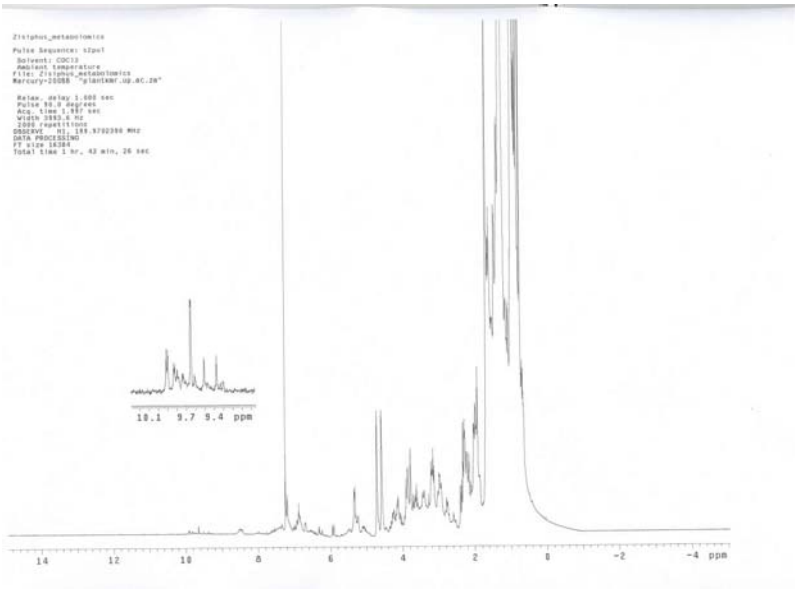


Fig. 3.12. ^{1}H -NMR spectrum of *Zisiphus mucronata.*

The samples of *D. rotundifolia* and *F. vulgare* contained characteristic doublets at 7.6 ppm and 6.25 ppm with the coupling constant for *D. rotundifolia* 9.4 Hz and for *F. vulgare* 8.4 Hz, which is characteristic of coumarins. They seem to be very similar compounds but indeed two different coumarins. The other two samples, *E. capensis and Z. mucronata*, also contained this type of compound, but in a smaller quantity which is undetectable on NMR.

3.3.4 Fingerprinting *Drosera capensis*

Table 3.3 gives the concentrations of 7-methyljuglone in the different plant parts tested on HPLC in *D. capensis.* Each sample were injected three times.

Table 3.3. Concentration of (10) in the different plant parts of *D. capensis*.

Plant part	[7-MJ] mg/g (wet mass)	[7-MJ] mg/g (dry mass)
Flower	9.63 ± 0.06	56.60 ± 0.06
Flower stem	1.11 ± 0.03	6.44 ± 0.03
Leaf lamina	1.84 ± 0.03	14.08 ± 0.10
Leaf petiole	1.31 ± 0.02	12.10 ± 0.06
Roots	3.14 ± 0.04	17.04 ± 0.11

The high amount of 7-methyljuglone in the flowers suggests that the compound (which is responsible for the red/orange colour) might have a functional role such as a pollinator attractant. Reports that these naphthoquinones act as antifeedant compounds might also be possible (Tokunaga *et al.*, 2004). The dimeric forms of 7-methyljuglone, neodiospyrin and mamegakinone, could positively be identified with HPLC (authentic standards) and the proton hydroxy shifts (Fig. 3.13) (Lillie & Musgrave, 1977). Diospyrin and isodiospyrin could up to now only be identified with HPLC. This will however be investigated further with NMR analysis using larger sample sizes.

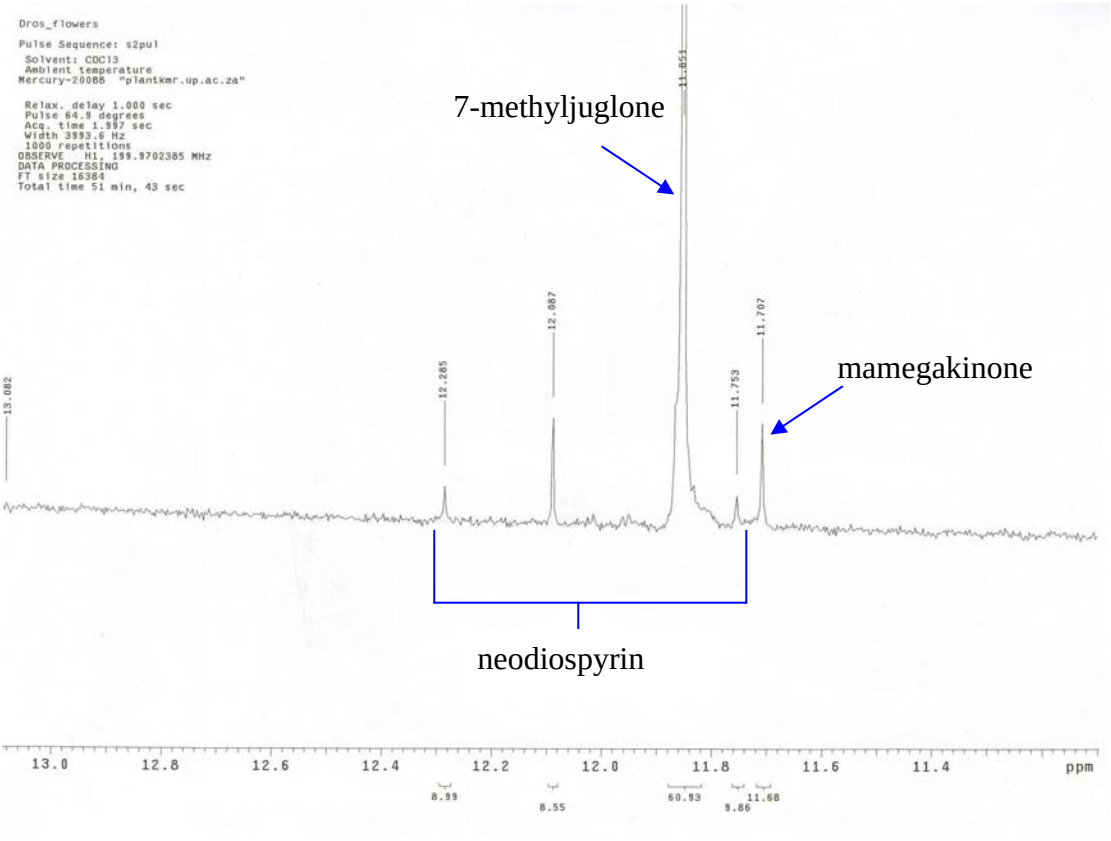


Fig. 3.13.The ^{1}H-NMR spectrum of the hydroxyl region confirming the identity of the compounds.

3.4 Discussion and conclusions

The main aim of this chapter was to determine if there is a link between plants used to treat TB related ailments and specific compounds in the extracts. TLC, HPLC and NMR analysis was therefore employed to confirm the precense of naphthoquinones (because they show good activity against TB) or any other class of compounds that might indicate that this link exists.

The limited analysis that was performed during this chapter indicated that the naphthoquinones are indeed only limited to very specific families of plants. 7-Methyljuglone could only be found in one species namely *D. capensis*. The use of an HPLC system confirmed that no naphthoquinones were present in any of the other plant material. HPLC is a very useful analytical tool in this research field. Some improvements in the setup is however required. The absence of a degasser destabilises the baseline and therefore no gradient mobile phase could be used. During this study the HPLC could be employed to analyse the samples for the presence of naphthoquinones, but not to fingerprint the whole extract with the use of a gradient system.

The analysis that was performed on the NMR did indeed give some correlation between some of the spectra. To be able to get a more reliable result, primary metabolites such as chlorophyll should be subtracted from the spectra. This will give a clearer picture of the secondary metabolites in the extracts. The complexity of the spectra makes it difficult to compare. Region 1 (0-2.5ppm) which will contain chlorophyll, terpenoids, apolar fats and hydrocarbons was too complex to compare. The solvent that was used was expected to extract a major amount of these compounds. The presence of coumarins on TLC and NMR shows that this method of profiling might yield useful information on active compounds in extracts. The presence of similar compounds (e.g. coumarins) indicates that this might be a biologically active compound. Previous reports on coumarins suggest that they interfere with Men enzymes responsible for the production of the mycobacterial menaquinone (Dialameh, 1978). Only *D. capensis* contained naphthoquinones and for the first time the dimeric forms of these compounds has been detected in this species. New software have been developed which would make NMR comparison much more accurate and faster. This software (AMIX vers. 6.1) subdivides the obtained spectra in small intergral regions (0.04 ppm). This is also known as bucketing. These regions are expressed in a bucket table which are then analysed with statistical software (SIMPCA-P). The end result is that differences between spectra are highlighted or the comparisons between the samples will group the samples together. The specific compound(s) which causes the grouping can then be further investigated and identified. The required software and the techniques will be investigated during further studies.

3.5. References

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