The medicinal and chemical aspects of naphthoquinones isolated from *Euclea natalensis* A. DC. on *Mycobacterium tuberculosis*

by

Frank van der Kooy

Submitted in partial fulfillment of the requirements for the degree of Philosophiae Doctor

In the Faculty of Natural and Agricultural Sciences
Department of Botany
University of Pretoria

Pretoria
January 2007

Promoter: Prof. J.J.M. Meyer

The medicinal and chemical aspects of naphthoquinones isolated from *Euclea natalensis* A. DC. on *Mycobacterium tuberculosis*

by

Frank van der Kooy

Supervisor: Prof. J.J.M. Meyer

Department: Botany

Degree: Philosophiae Doctor

Abstract

The isolation and antimycobacterial activity of several naphthoquinones from *Euclea natalensis* were previously reported and initiated this study into the occurrence, chemistry and biological activity of this class of compounds. The structure activity relationship of the isolated naphthoquinones, and commercially available derivatives were also studied.

Several plant species were investigated to establish a possible link between their traditional use for chest related symptoms (including tuberculosis infection) and the occurrence of 7-methyljuglone in these plants. The plants were extracted and tested qualitatively with the use of three analytical tools for the presence of 7-methyljuglone or related naphthoquinones.

Due to its commercial unavailability, the chemical synthesis of two of these naphthoquinones, 7-methyljuglone and diospyrin, was attempted with varying degrees of success. The Friedel-Crafts acylation method was used to synthesise 7-methyljuglone from *m*-cresol and maleic anhydride as starting material. The optimisation of the synthesis was also investigated. Through a two-step pathway of epoxidation and steam distillation, diospyrin was

successfully synthesised albeit in small quantities. During the attempts to synthesise diospyrin, two other related compounds were also synthesised. These compounds, neodiospyrin and mamegakinone, are structural isomers of diospyrin.

The stability of some of the naphthoquinones was tested in various carriers in an attempt to explain the influence this will have on the obtained antituberculosis and toxicity data. The BACTEC vial solution, which is widely used to determine potency against *Mycobacterium tuberculosis*, was analysed with HPLC to determine the stability of these compounds in it. In addition the stability in organic solvents especially DMSO, was also tested as this is the solvent of choice for hydrophobic compounds in almost all bioassays.

The antituberculosis activity and/or toxicity of 7-methyljuglone was investigated with three bioassays, to broaden our knowledge on the mechanism of action of naphthoquinones. Vero cells were employed to determine the inhibitory concentration (IC50) of most of the naphthoquinones. Mice experiments were carried out to determine the toxicity of 7-methyljuglone and diospyrin *in vivo*. In addition the lead compound, 7-methyljuglone, was tested on *Musca domestica* (house fly) to establish its toxicity on this organism.

In order to find the pharmacophore of this class of compounds, a preliminary structure-activity relationship was conducted. During this study the active site in the compounds which confers potency and toxicity was partly established.

The mode of action of some of the naphthoquinones was investigated and it was established that the compounds might interfere with the mycobacterial electron transport chain. A fluorinated 7-methyljuglone stops the production of menaquinone which transports electrons from the NADH dehydrogenase complex to the cytochrome bc complex and effectively kills the mycobacterium.

Keywords: diospyrin, electron transport chain, 7-methyljuglone, *Mycobacterium tuberculosis,* structure-activity relationship.

TABLE OF CONTENTS

Chapter 1

Introduction

1.1	**General background and introduction**	**11**
1.1.1	**Occurrence and treatment of *Mycobacterium tuberculosis***	**11**
1.1.2	**Natural product chemistry**	**13**
1.1.3	**Organic synthesis**	**15**
1.1.4	**Stability and solubility of naphthoquinones**	**16**
1.1.5	**Toxicity of naphthoquinones**	**17**
1.1.6	**Structure-activity relationship**	**17**
1.1.7	**Mode of action studies**	**18**
1.2	**Objectives of this study**	**18**
1.3	**Structure of thesis**	**19**
1.4	**References**	**21**

Chapter 2

Literature review

2.1	**An introduction to *Euclea natalensis***	**24**
2.1.1	**Traditional uses**	**25**
2.1.2	**Phytochemistry**	**25**
2.2	**Occurrence and profiling of 7-methyljuglone in plants**	**26**
2.3	**Chemistry and biological activity of naphthoquinones**	**27**
2.3.1	**Synthesis of naphthoquinones**	**27**
2.3.2	**Biological activity of naphthoquinones**	**28**
2.3.3	**Mode of action of naphthoquinones**	**30**
2.4	**References**	**31**

Chapter 3

The occurrence and profiling of naphthoquinones in ethnobotanically selected plants

3.1 Introduction 39

3.2 Materials and methods 41

3.2.1 Plant material 41

3.2.2 Preparation of extracts 41

3.2.3 Profiling with TLC 42

3.2.4 Profiling with HPLC 42

3.2.5 Profiling with NMR 42

3.2.6 Fingerprinting *Drosera capensis* 43

3.3 Results 43

3.3.1 Profiling with TLC 43

3.3.2 Profiling with HPLC 45

3.3.3 Profiling with NMR 45

3.3.4 Fingerprinting *Drosera capensis* 50

3.4 Discussion and conclusions 51

3.5 References 53

Chapter 4

Synthesis of 7-methyljuglone and diospyrin

4.1 Introduction 57

4.2 Materials and methods 58

4.2.1 Materials 58

4.2.2 Methods 59

4.2.2.1 Synthesis of 7-methyljuglone 59

4.2.2.1.1 Effect of different quantities of reagents on 7-methyljuglone formation 59

4.2.2.1.2 Effect of different quantities of catalyst on 7-methyljuglone formation 60

4.2.2.1.3 Influence of temperature on 7-methyljuglone formation 60

4.2.2.1.4	*Effect of altering reaction times*	*61*
4.2.2.2	**Epoxidation of 7-methyljuglone**	**61**
4.2.2.2.1	*Effect of reaction time on epoxide formation*	*61*
4.2.2.2.2	*Influence of time before acidification*	*61*
4.2.2.2.3	*Effect of amount of acid on epoxide formation*	*62*
4.2.2.3	**Synthesis of diospyrin**	**62**
4.2.2.3.1	*Oxidative dimerisation of 7-methyljuglone*	**62**
4.2.2.3.2	*Buffered reaction between hydroquinone and 7-methyljuglone*	**62**
4.2.2.3.3	*Epoxide condensation*	**63**
4.2.2.3.3.1.	*Addition of an Bronsted-Lowry acid to the epoxide*	*63*
4.2.2.3.3.2	*Addition of an Lewis acid and steam distillation*	*63*
4.3	**Results**	**64**
4.3.1	**Synthesis of 7-methyljuglone**	**64**
4.3.1.1	**Effect of different quantities of reagents on 7-methyljuglone formation**	**64**
4.3.1.2	**Effect of different catalyst ratios**	**64**
4.2.1.3	**Influence of temperature on 7-methyljuglone formation**	**65**
4.1.2.4	**Effect of altering stirring times**	**65**
4.3.2	**Epoxidation of 7-methyljuglone**	**66**
4.3.2.1	**Influence of reaction time on epoxide formation**	**66**
4.3.2.2	**Effect of time before acidification**	**66**
4.3.2.3	**Effect of the amount of acid**	**67**
4.3.3	**Synthesis of diospyrin**	**68**
4.3.3.1	**Oxidative dimerisation of 7-methyljuglone**	**68**
4.3.3.2	**Buffered reaction between hydroquinone and 7-methyljuglone**	**68**
4.3.2.3	**Epoxide condensation**	**68**
4.3.2.3.1	*Addition of Bronsted-Lowry acid*	*68*
4.3.2.3.2	*Addition of Lewis acid and steam distillation*	*69*
4.4	**Discussion and conclusions**	**70**
4.5	**References**	**74**

Chapter 5

Stability of naphthoquinones

5.1 Introduction 76

5.2 Materials and methods 77

5.2.1 Materials 77

5.2.2 Methods 77

5.2.2.1 Stability in dimethylsulfoxide 77

5.2.2.2 Stability in BACTEC buffer solution 78

5.2.2.3 Stability in vero cell toxicity bioassay buffer 78

5.2.2.4 Stability in 20% DMSO/Agar mixture 78

5.2.2.5 Stability in broth used for mode of action studies 78

5.3 Results 79

5.3.1 Stability in dimethylsulfoxide 79

5.3.2 Stability in BACTEC buffer solution 82

5.3.3 Stability in vero cell toxicity bioassay buffer 83

5.3.4 Stability in 20% DMSO/Agar mixture 84

5.3.5 Stability in broth used for mode of action studies 84

5.4 Discussion and conclusions 84

5.5 References 87

Chapter 6

Toxicity of naphthoquinones

6.1 Introduction 89

6.2 Materials and methods 89

6.2.1 Materials 89

6.2.1.1 Culturing of Vero monkey kidney cells 89

6.2.1.2 Toxicity of 7-methyljuglone and diospyrin in mice 90

6.2.1.3 Toxicity of 7-methyljuglone in *Musca domestica* 90

6.2.2 Methods 90

6.2.2.1 Determination of the IC50 of naphthoquinones on vero cells 90

6.2.2.2 Toxicity of 7-methyljuglone and diospyrin in mice 91

6.2.2.3 Toxicity of 7-methyljuglone in *Musca domestica* 91

6.3 Results 91

6.3.1 Determination of the IC50 of naphthoquinones on vero cells 91

6.3.2 Toxicity of 7-methyljuglone and diospyrin in mice 92

6.3.3 Toxicity of 7-methyljuglone in *Musca domestica* 93

6.4 Discussion and conclusions 95

6.5 References 98

Chapter 7

Structure-activity relationship of naphthoquinones

7.1 Introduction 100

7.2 Materials and methods 101

7.2.1 MIC determination 101

7.2.2 Toxicity bioassay 103

7.3 Results 103

7.3.1 MIC and toxicity determination 103

7.3.2 Structure-activity relationship 104

7.4 Discussion and conclusions 105

7.5 References 106

Chapter 8

The mode of action of naphthoquinones in *Mycobacterium smegmatis*

8.1 Introduction 108

8.2 Materials and methods **113**

8.2.1 Activity against *M. smegmatis* 113

8.2.2 *M. smegamatis* cultures 113

8.2.3 Extraction of M. smegmatis cells 114

8.2.4 HPLC analysis 114

8.3 Results **114**

8.3.1 Activity against *M. smegmatis* 114

8.3.2 *M. smegamatis* cultures 116

8.3.3 HPLC analysis 116

8.4 Discussion and conclusions **118**

8.5 References **121**

Chapter 9 **123**

General Discussion and Conclusions

Acknowledgements and Publications **127**

Chapter 1

Introduction

1.1 General background and introduction 11

1.1.1 Occurrence and treatment of *Mycobacterium tuberculosis* 11

1.1.2 Natural product chemistry 13

1.1.3 Organic synthesis 15

1.1.4 Stability and solubility of naphthoquinones 16

1.1.5 Toxicity of naphthoquinones 17

1.1.6 Structure-activity relationship 17

1.1.7 Mode of action studies 18

1.2 Objectives of this study 18

1.3 Structure of thesis 19

1.4 References 21

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).

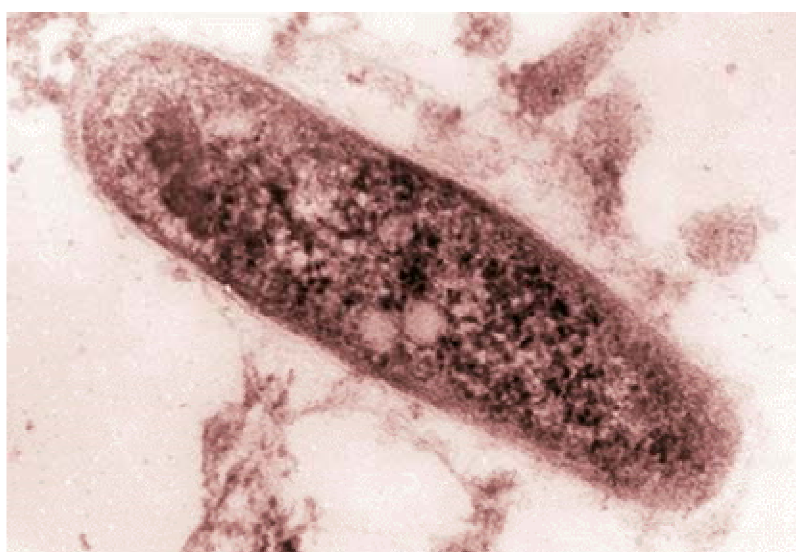


Fig. 1.1: Electron microscope image of *M. tuberculosis* (http://www.abc.net.au/science/news/img/tb.jpg)

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.

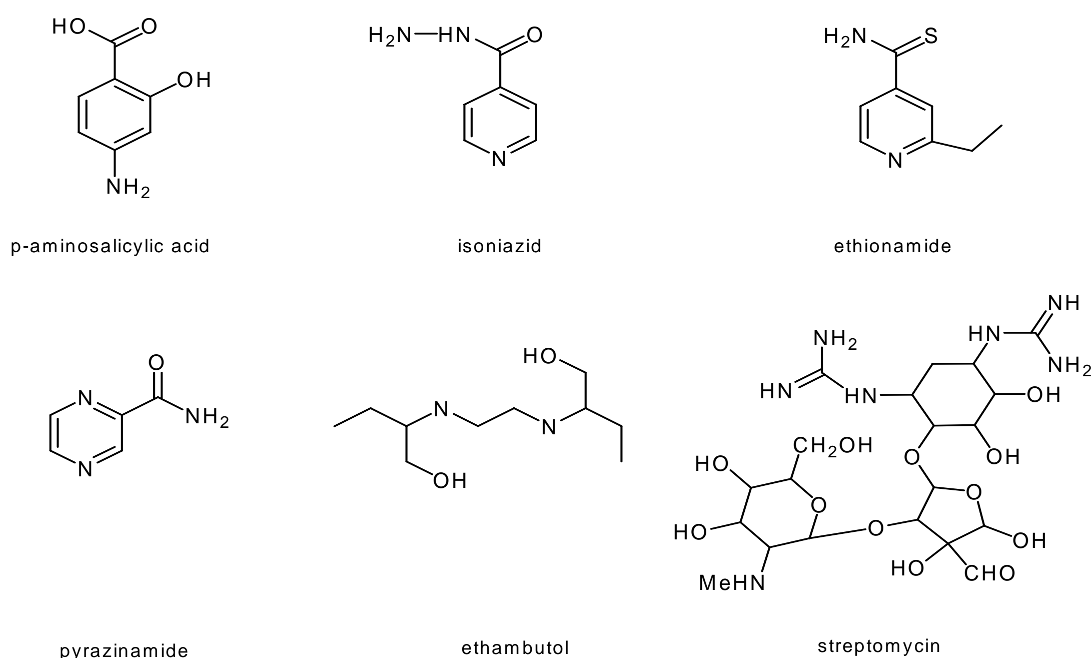


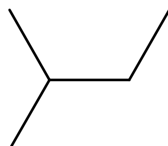
Fig. 1.2: Structures of some antimycobacterial drugs (Young, 1994)

1.1.2 Natural product chemistry

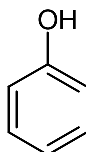
Natural product chemistry or the research into secondary metabolites from higher plants and other organisms has been conducted for centuries. Plants produce a large and diverse array of organic compounds that appear to have no direct function in growth and development (Taiz & Zeiger, 2002). These substances 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:

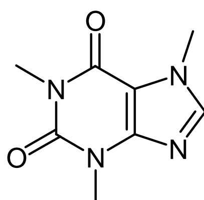
a) Terpenes – consisting of isopentane units (5-carbon elements).



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.

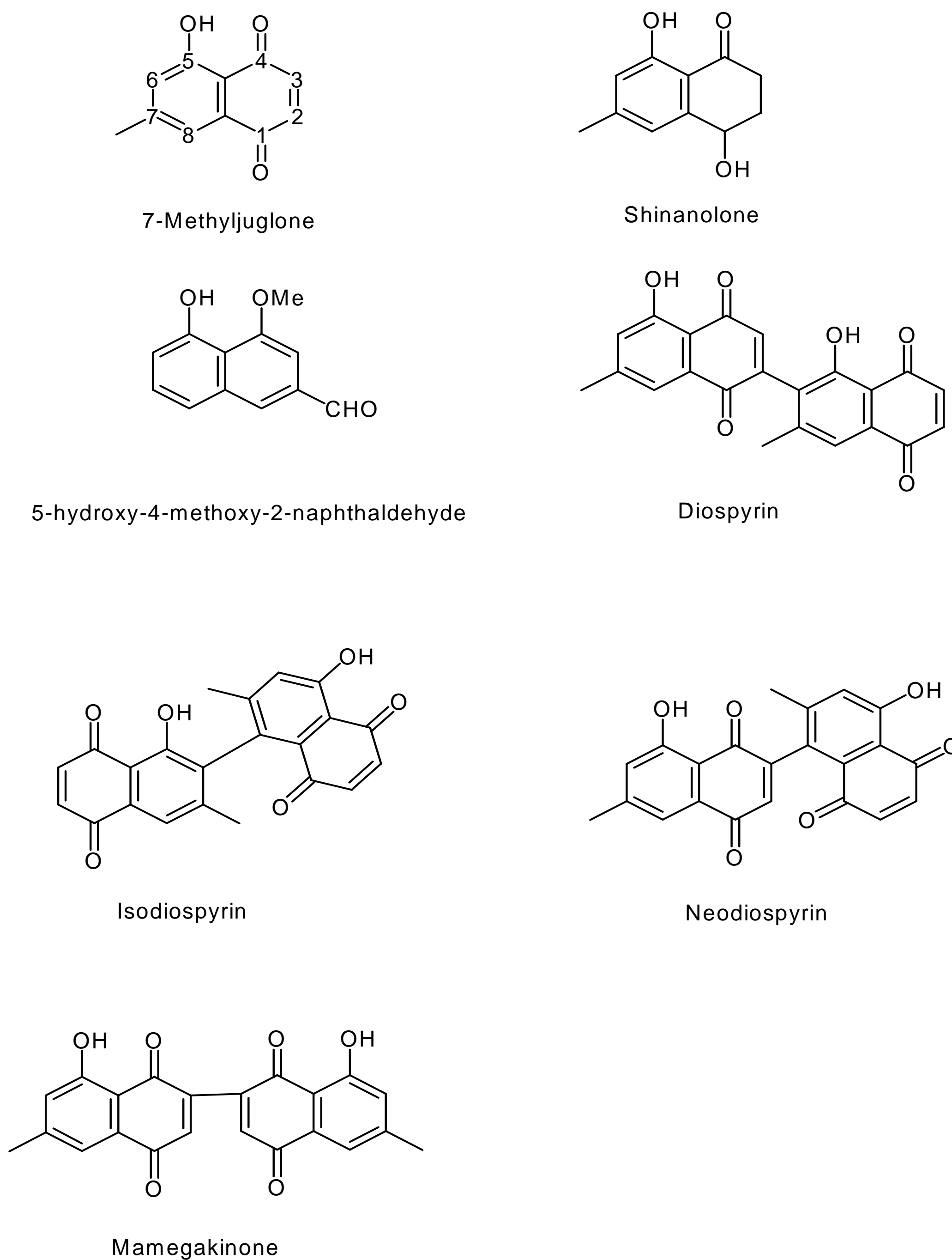


Fig. 1.3: Compounds previously isolated by the author from *Euclea natalensis* (Van der Kooy, 2003). The numbering system is indicated for 7-methyljuglone.

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

Chapman & Hall/CRC. (2006). Dictionary of Natural Products. Vol 12:3. HDS Software copyright © Hampden Data Services Ltd.

Evans, C.C. (1998). Historical background. In: Clinical tuberculosis, ed. P.D.O. Davies, pp. 3,17. Chapman & Hall Medical, London.

Global Alliance for TB Drug Development. (2005). pp1-3. Broad Street, 31st floor, New York, US.

Lall, N. & Meyer, J.J.M. (2000). Antibacterial activity of water and acetone extracts of the roots of *Euclea natalensis. Journal of Ethnopharmacology.* 72: 313-316.

Lehman, J. (1946). Para-aminosalicylic acid in the treatment of tuberculosis. *The Lancet.* 247: 15-16.

Mallavadhani, U.V., Panda, A.K. & Rao, Y.R. (1998). Pharmacology and chemotaxonomy of *Diospyros*. *Phytochemistry.* 49: 901-951.

McMurry, J. (1996). Organic chemistry. 4th ed. pp 1-3. Brookes/Cole Publishing, USA.

Morrison, R. T. & Boyd, R. N. (1992). Organic chemistry. 6th ed. pp 1-3, 666, 901, 764, 905. Prentice Hall International, Inc.

Schatz, A. & Waksman, S.A. (1944). Effect of streptomycin and other antibiotic substances upon *Mycobacterium tuberculosis* and related organisms. *Proceedings of the Society for Experimental Biology and Medicine.* 57: 244-245.

Silverman, R.B. (2004). The organic chemistry of drug design and drug action. pp 21-22, Elsevier Academic Press, USA.

Taiz, L. & Zeiger, E. (2002). Plant defences: Surface protectants and secondary metabolites. In: Plant Physiology, 3ed, Ch. 13. pp 349-350. Sinauer Associates, Inc. Sunderland, Massachusetts.

Timbrell, J.A. (1991). Principles of biochemical toxicology. 2nd ed. pp 7-9. Taylor & Francis, London.

Van der Kooy, F. (2003). Characterisation, synthesis and antimycobacterial activity of naphthoquinones isolated from *Euclea natalensis.* Unpublished. M.Sc. dissertation. University of Pretoria. South Africa.

Young, D.B. (1994). Strategies for new drug development. In: Clinical tuberculosis, ed. P.D.O. Davies, pp.3,17. Chapman & Hall Medical, London.

Chapter 2

Literature review

2.1 An introduction to *Euclea natalensis* **24**

2.1.1 Traditional uses **25**

2.1.2 Phytochemistry **25**

2.2 Occurrence and profiling of 7-methyljuglone in plants **26**

2.3 Chemistry and biological activity of naphthoquinones **27**

2.3.1 Synthesis of naphthoquinones **27**

2.3.2 Biological activity of naphthoquinones **28**

2.3.3 Mode of action of naphthoquinones **30**

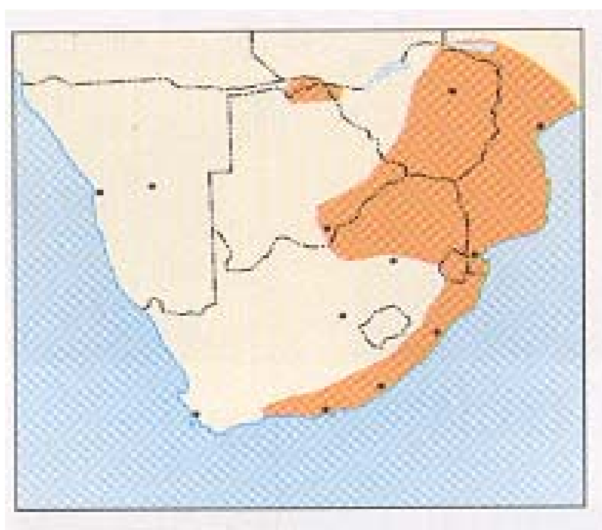
2.4 References **31**

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

University of Pretoria etd – Van der Kooy, F (2007)

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

Adeniyi, B. A., Fong, H. H. S., Pezzuto, J. M., Luyengi, L. & Odelola, H. A. (2000). Antibacterial activity of diospyrin , isodiospyrin and bisisodiospyrin from the root of *Diospyros piscatoria* (Gurke) (Ebenaceae). *Phytotherapy Research.* 14(2), 112-117.

Bailly, C. (2000). Topoisomerase I poisons and suppressors as anticancer drugs. *Current Medicinal Chemistry*. 7(1), 39-58.

Bohm, P. A. F., Zanardo, F. M. L., Ferrarese, M. L. L. & Ferrarese-Filho, O. (2006). Peroxidase activity and lignification in soybean root growth - inhibition by juglone. *Biologia Plantarum*. 50(2), 315-317.

Boisvert, L. & Brassard, P. (1988). Regiospecific addition of monooxygenated dienes to halo quinines. *Canadian Journal of Organic Chemistry*. 53(17), 4052-9.

Brockmann, H. & Laatsch, H. (1983). Regioselective syntheses of 3,3'-bijuglone, mamegakinone, dianellinone, cyclo-trijuglone, xylospyrin, and trianellinone by phenol-quinone addition. *Liebigs Annalen der Chemie.* (3), 433-47.

Caniato, R., Filippini, R., & Cappelletti, E. M. (1989). Naphthoquinone contents of cultivated *Drosera* species *Drosera binata*, *D. binata* var. *dichotoma*, and *D. capensis*. *International Journal of Crude Drug Research.* 27(3), 129-36.

Carter, F.L., Garlo, A.M., & Stanley, J.B. (1978). Termicidal components of wood extracts: 7-methyljuglone from *Diospyros virginiana*. *Journal of Agriculture and Food Chemistry*. 26(4), 869-73.

Chapman & Hall/CRC. (2006). Dictionary of Natural Products. Vol 12:3. HDS Software copyright © Hampden Data Services Ltd.

Cooke, R.G. & Dowd, H. (1952). Colouring matters of Australian plants. III. Synthesis of 7-methyljuglone and related compounds. *Australian Journal of Chemistry.* 1: 53-57.

Cushion, M. T., Collins, M., Hazra, B. & Kaneshiro, E. S. (2000). Effects of atovaquone and diospyrin-based drugs on the cellular ATP of *Pneumocystis carinii* f. sp. *carinii. Antimicrobial Agents and Chemotherapy.* 44(3), 713-719.

Evans, C.C. (1998). Historical background. In: Clinical tuberculosis, ed. P.D.O. Davies, pp. 3,17. Chapman & Hall Medical, London.

Gafner, F. & Rodriguez, E. (1989). Biological chemistry of molluscicidal and cytotoxic plants constituents. *Revista Latinoamericana de Quimica*. 20(1), 30-1.

Ganapaty, S., Pannakal, S.T., Fotso, S & Laatsch, H. (2004). Antitermitic quinones from *Diospyros sylvatica*. *Phytochemistry.* 65(9) 1265-1271.

Gu, J., Graf, T. N., Lee, D., Chai, H., Mi, Q., Kardono, L. B. S., Setyowati, F. M., Ismail, R., Riswan, S., Farnsworth, N. R., Cordell, G. A., Pezzuto, J. M., Swanson, S. M., Kroll, D. J., Falkinham, J. O., Wall, M. E., Wani, M. C., Kinghorn, A. D. & Oberlies, N H. (2004). Cytotoxic and Antimicrobial Constituents of the Bark of *Diospyros maritima* Collected in Two Geographical Locations in Indonesia. *Journal of Natural Products.* 67(7), 1156-1161.

Hazra, B., Kumar, B., Biswas, S., Pandey, B.N. & Mishra, K.P. (2005). Enhancement of the tumor inhibitory activity, in vivo, of diospyrin, a plantderived quinonoid, through liposomal encapsulation. *Toxicology Letters.* 157(2) 109-117.

Hazra, B., Golenser, J., Nechemiya, O., Bhattacharyya, S., Azzam, T., Domb, A. & Frankenburg, S. (2002). Inhibitory activity of diospyrin derivatives against Leishmania major parasites in vitro. *Indian Journal of Pharmacology*. 34(6), 422-427.

Ito, Y., Hayashi, Y. & Kato, A. (1995). Antifungal compounds from trees of the genus *Diospyros* with complete assignment of nuclear magnetic resonance data. *Mokuzai Gakkaishi.* 41(7), 694-8.

Jordaan, M. (2003). Ebenaceae. In G. Germishuizen & N.L. Meyer (eds), Plants of southern Africa: an annotated checklist. Sterlitzia 14: 421-423. National Botanical Institute, Pretoria.

Kapadia, G. J., Azuine, M. A., Balasubramanian, V. & Sridhar, R. (2001). Aminonaphthoquinones-a novel class of compounds with potent antimalarial activity against *Plasmodium falciparum*. *Pharmacological Research*. 43(4), 363-367.

Kayser, O., Kiderlen, A. F., Laatsch, H. & Croft, S. L. (2000). In vitro leishmanicidal activity of monomeric and dimeric naphthoquinones. *Acta Tropica.* 77(3), 307-314.

Khalafy, J. & Bruce, J. M. (2002). Oxidative dehydrogenation of 1-tetralones: Synthesis of juglone, naphthazarin, and hydroxyanthraquinones. *Journal of Sciences, Islamic Republic of Iran.* 13(2), 131-139.

Kuke, C., Williamson, E. M., Roberts, M. F., Watt, R., Hazra, B., Lajubutu, B. A. & Yang, S. (1998). Antiinflammatory activity of binaphthaquinones from *Diospyros* species. *Phytotherapy Research.* 12(3), 155-158.

Kumari, L. K., Babu, M. H. & Pardhasaradhi, M. (1982). Synthesis of neodiospyrin and fixation of aryl-quinone linkage in its structure. *Indian Journal of Chemistry, Section B: Organic Chemistry Including Medicinal Chemistry.* 21B(7), 619-21.

Lall, N. & Meyer J.J.M. (1999). In vitro inhibition of drug-resistant and drug-sensitive strains of *Mycobacterium tuberculosis* by ethnobotanically selected South African plants. *Journal of Ethnopharmacology.* 66(3), 347-54.

Lall, N. & Meyer, J. J. M. (2001). Inhibition of drug-sensitive and drug-resistant strains of *Mycobacterium tuberculosis* by diospyrin, isolated from *Euclea natalensis*. *Journal of Ethnopharmacology*. 78(2-3), 213-216.

Lall, N., Das Sarma, M., Hazra, B. & Meyer, J. J. M. (2003). Antimycobacterial activity of diospyrin derivatives and a structural analogue of diospyrin against *Mycobacterium tuberculosis* in vitro. *Journal of Antimicrobial Chemotherapy*. 51(2), 435-438.

Lall, N., Meyer, J. J. M., Wang, Y., Bapela, N. B., van Rensburg, C. E. J., Fourie, B. & Franzblau, S. G. (2005). Characterization of intracellular activity of antitubercular

constituents from the roots of *Euclea natalensis*. *Pharmaceutical Biology (Philadelphia, PA, United States).* 43(4), 353-357.

Lebrasseur, N., Fan, G., Oxoby, M., Looney, M.A. & Quideau, S. (2005). 3-Iodane-mediated arenol dearomatization. Synthesis of five-membered ring-containing analogues of the aquayamycin ABC tricyclic unit and novel access to the apoptosis inducer menadione. *Tetrahedron.* 61(6), 1551-1562.

Lee, K. & Campbell, R.W. (1969). Nature and occurrence of juglone in *Juglans nigra*. *HortScience*. 4(4), 297-8.

Li, H. H., Nishimura, H., Koji, H. & Mizutani, J. (1993). Some physiological effects and the possible mechanism of action of juglone in plants. *Zasso Kenkyu.* 38(3), 214-22.

Likhitwitayawuid, K., Dej-Adisai, S., Jongbunprasert, V. & Krungkrai, J. (1999). Antimalarials from *Stephania venosa*, *Prismatomeris sessiliflora*, *Diospyros montana*, and *Murraya siamensis*. *Planta Medica.* 65(8), 754-756.

Mallavadhani, U.V., Panda, A.K. & Rao, Y.R. (1998). Pharmacology and chemotaxonomy of *Diospyros*. *Phytochemistry.* 49: 901-951.

Marston, A., Msonthi, J. D. & Hostettmann, K. (1984). Phytochemistry of African medicinal plants. Part 1. Naphthoquinones of *Diospyros usambarensis*; their molluscicidal and fungicidal activities. *Planta Medica.* 50(3), 279-80.

Marczak, L., Kawiak, A., Lojkowska, E. & Stobiecki, M. (2005). Secondary metabolites in in vitro cultured plants of the genus Drosera. *Phytochemical Analysis*. 16(3), 143-149.

Musgrave, O.C. & Skoyles, D. (2001). Ebenaceae extractives. Part11. The synthesis of 7-methyljuglone. A re-examination. *Journal of the Chemical Society. Perkin Transactions*. 1: 1318-1320.

Neuhaus-Carlisle, K., Vierling, W. & Wagner, H. (1997). Screening of plant extracts and plant constituents for calcium-channel blocking activity. *Phytomedicine.* 4(1), 67-71.

Park, B., Lee, H., Lee, S., Piao, X., Takeoka, G. R., Wong, R. Y., Ahn, Y. & Kim, J. (2006). Antibacterial activity of *Tabebuia impetiginosa* Martius ex DC (Taheebo) against *Helicobacter pylori*. *Journal of Ethnopharmacology*. 105(1-2), 255-262.

Ragazzi, E., De Biasi, M. Pandolfo, L. Chinellato, A. & Caparrotta, L. (1993). *In vitro* effects of naphthoquinones isolated from *Drosera* species. *Pharmacological Research* 27, 87-88.

Sparg S G., Van Staden, J. & Jager, A.K. (2000). Efficiency of traditionally used South African plants against schistosomiasis. *Journal of Ethnopharmacology.* 73(1-2), 209-14.

Stander, I. & Van Wyk, C.W. (1991). Toothbrushing with the root of *Euclea natalensis*. *Journal de Biologie Buccale.* 19: 167-172.

Steffen, K. & Peschel, H. (1975). Chemical constitution and antifungal activity of 1,4-naphthoquinones, their biosynthetic intermediates, and chemically related compounds. *Planta Medica*. 27(3), 201-12.

Suzuki, T., Haga, K., Kataoka, M., Tsutsumi, T., Nakano, Y., Matsuyama, S. & Kuwahara, Y. (1995). Secretion of thrips. VIII. Secretions of the two *Ponticulothrips* species (Thysanoptera: Phlaeothripidae). *Applied Entomology and Zoology.* 30(4), 509-19.

Tallman, E.A. (1984). Part I. Annelative phenol synthesis. Synthesis of 7-methyljuglone and 11-deoxydaunomycinone. Unpublished, M.Sc. dissertation. Brown University, Providence, RI, USA.

Tazi, J., Bakkour, N., Soret, J., Zekri, L., Hazra, B., Laine, W., Baldeyrou, B., Lansiaux, A. & Bailly, C. (2005). Selective inhibition of topoisomerase I and various steps of spliceosome assembly by diospyrin derivatives. *Molecular Pharmacology.* 67(4), 1186-1194.

Tikkanen, L., Matsushima, T. Natori, S. & Yoshihira, K. (1983). Mutagenicity of natural naphthoquinones and benzoquinones in the Salmonella/microsome test. *Mutation Research*. 124(1), 25-34.

Timothy, F. (1995). Novel quinone antiproliferate inhibitors of phosphatidylinositol-3-kinase. *Anti-cancer Drug Design*. 10(4), 347-59.

Ting, C., Hsu, C., Hsu, H., Su, J., Chen, T., Tarn, W., Kuo, Y., Whang-Peng, J., Liu, L. F. & Hwang, J. (2003). Isodiospyrin as a novel human DNA topoisomerase I inhibitor. *Biochemical Pharmacology*. 66(10), 1981-1991.

Tokunaga, T., Takada, N & Ueda, M. (2004). Mechanism of antifeedant activity of plumbagin, a compound concerning the chemical defence in carnivorous plants. *Tetrahedron letters*. 45(38), 7115-7119.

Tomaszkiewicz-Potepa, A & Vogt, O. (2004). Juglone - a biologically active metabolite from plants of family Juglandaceae. *Wiadomosci Chemiczne*. 58(11-12), 881-894.

Tran, T., Saheba, E., Arcerio, A. V., Chavez, V., Li, Q., Martinez, L. E. & Primm, T.P. (2004) Quinones as antimycobacterial agents. *Bioorganic & Medicinal Chemistry*. 12(18), 4809-4813.

Van der Kooy, F. (2003). Characterisation, synthesis and antimycobacterial activity of naphthoquinones isolated from *Euclea natalensis.* Unpublished. M.Sc. dissertation. University of Pretoria. South Africa.

Van der Kooy, F., Meyer, J.J.M. & Lall, N. (2006). Antimycobacterial activity and possible mode of action of newly isolated neodiospyrin and other naphthoquinones from *Euclea natalensis. South African Journal of Botany*. 72: 349-352.

Van der Viyver, L.M. & Gerritsma, K.W. (1974). Naphthoquinones of *Euclea* and *Diospyros* species. *Phytochemistry*. 13: 2322-2323.

Van Wyk, B. & Van Wyk, P. (1997). Field guide to trees of Southern Africa, pp 184-185. Struik, McKenzie street, Cape Town.

Van Wyk BE., Van Oudshoorn, B. & Gericke, N. (2002). Medicinal plants of South Africa, pp110, 132, 290. Briza Publications, Arcadia, Pretoria.

Villas-Boas, S.G., Rasmussen, S. & Lane, G.A. (2005). Metabolomics or metabolite profiling. *Trends in biotechnology*. 23(8), 385-386.

Verrax, J., Bollen, S., Delvaux, M., Taper, H. & Calderon, P. (2005). New insights about the potential application of the association of vitamins C (sodium ascorbate) and K3 (menadione) as auxiliary therapy in cancer treatment. *Medicinal Chemistry Reviews*. 2(4), 277-282.

Wang, J., Burger, R. M. & Drlica, K. (1998). Role of superoxide in catalase-peroxidase-mediated isoniazid action against mycobacteria. *Antimicrobial Agents and Chemotherapy*. 42(3), 709-711.

Watt, J.M. & Breyer-Brandwijk, M.G. (1962) The medicinal and poisonous plants of southern and eastern Africa. 2nd edition. Livingstone, London, p390.

Weigenand, O., Hussein, A.A., Lall, N. & Meyer, J. J. M. (2004). Antibacterial Activity of Naphthoquinones and Triterpenoids from *Euclea natalensis* Root Bark. *Journal of Natural Products*. 67(11), 1936-1938.

Wube, A. A., Streit, B., Gibbons, S., Asres, K. & Bucar, F. (2005). In vitro 12(S)-HETE inhibitory activities of naphthoquinones isolated from the root bark of *Euclea racemosa* ssp. *schimperi*. *Journal of Ethnopharmacology*. 102(2), 191-196.

Yang, S. Y., Kim, H. K., Lefeber, A. W. M., Erkelens, C., Angelova, N., Choi, Y. H. & Verpoorte, R. (2006). Application of two-dimensional nuclear magnetic resonance spectroscopy to quality control of ginseng commercial products. *Planta Medica*. 72(4), 364-369.

Yoshida, M. & Mori, K. (2000). Synthesis of diospyrin, a potential agent against Leishmaniasis and related parasitic protozoan diseases. *European Journal of Organic Chemistry*. 1313-1317.

Chapter 3

The occurrence and profiling of naphthoquinones in ethnobotanically selected plants

3.1	**Introduction**	**39**
3.2	**Materials and methods**	**41**
3.2.1	**Plant material**	**41**
3.2.2	**Preparation of extracts**	**41**
3.2.3	**Profiling with TLC**	**42**
3.2.4	**Profiling with HPLC**	**42**
3.2.5	**Profiling with NMR**	**42**
3.2.6	**Fingerprinting *Drosera capensis***	**43**
3.3	**Results**	**43**
3.3.1	**Profiling with TLC**	**43**
3.3.2	**Profiling with HPLC**	**45**
3.3.3	**Profiling with NMR**	**45**
3.3.4	**Fingerprinting *Drosera capensis***	**50**
3.4	**Discussion and conclusions**	**51**
3.5	**References**	**53**

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

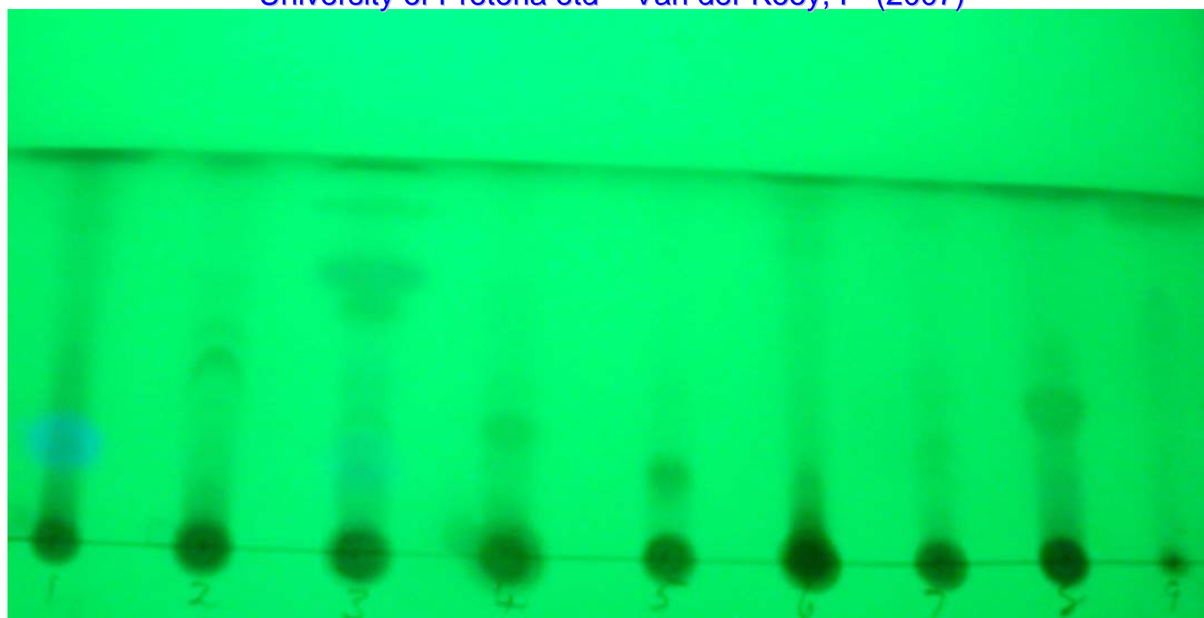
3.2.6 Fingerprinting *Drosera capensis*

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

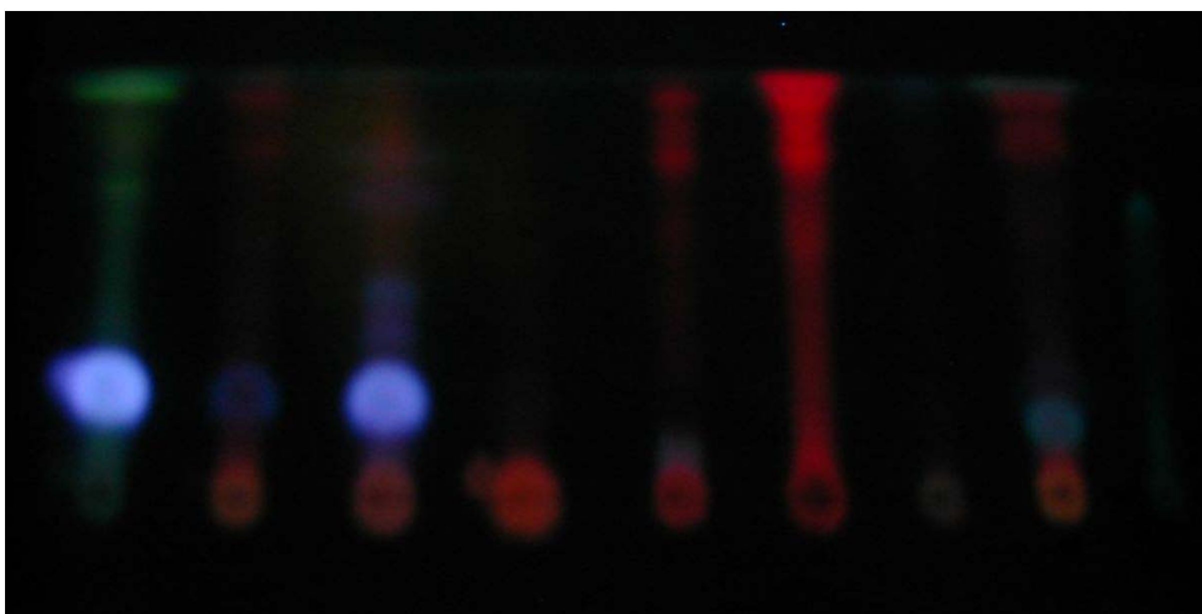
3.3.1 Profiling with TLC

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.

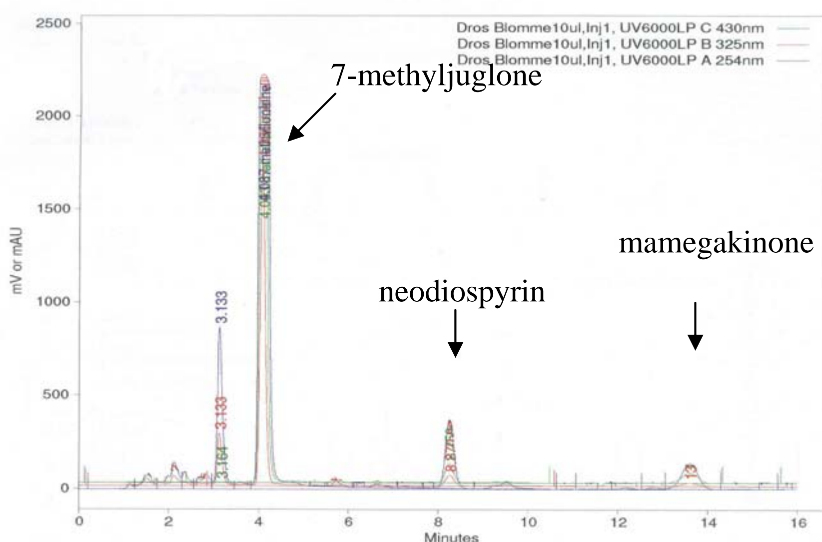


Fig. 3.3. HPLC chromatogram indicating the presence of 7-methyljuglone, neodiospyrin and mamegakinone in the *D. capensis* crude extract.

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.

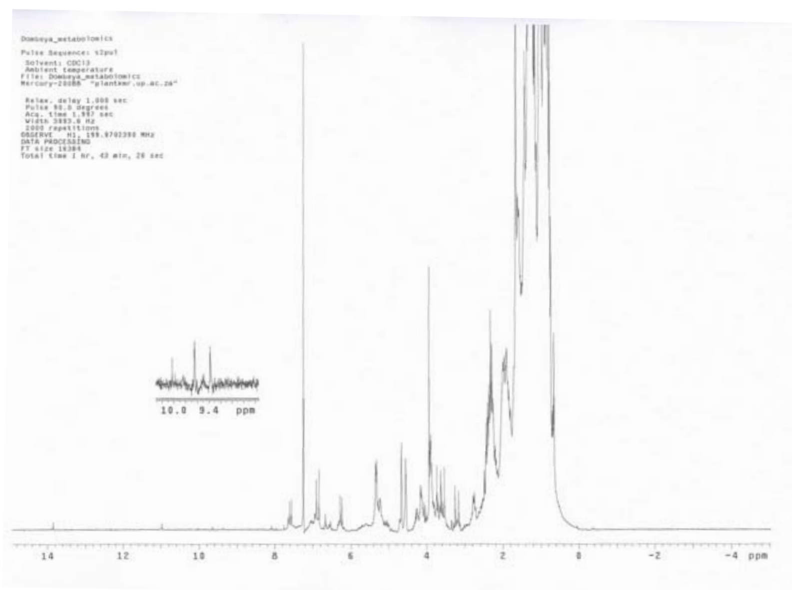


Fig. 3.4. The ^{1}H -NMR spectrum of *Dombeya rotundifolia*. The region between 9-10ppm is indicated in the inset.

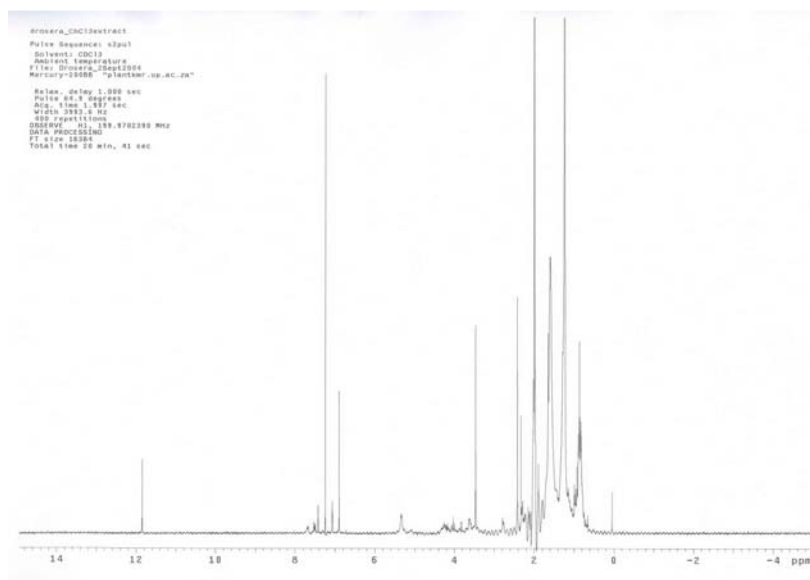


Fig. 3.5. The ^{1}H -NMR spectrum of *Drosera capensis.*

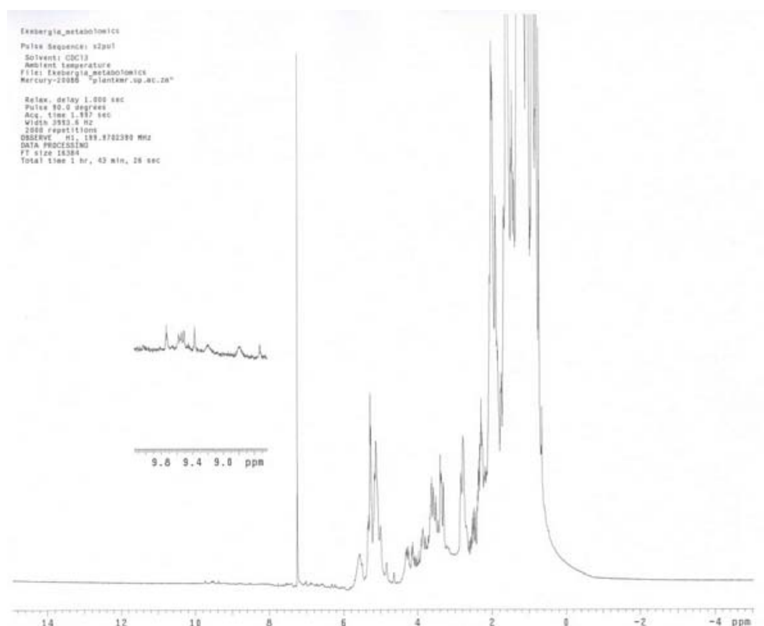


Fig. 3.6. ^{1}H -NMR spectrum of *Ekebergia capensis*.

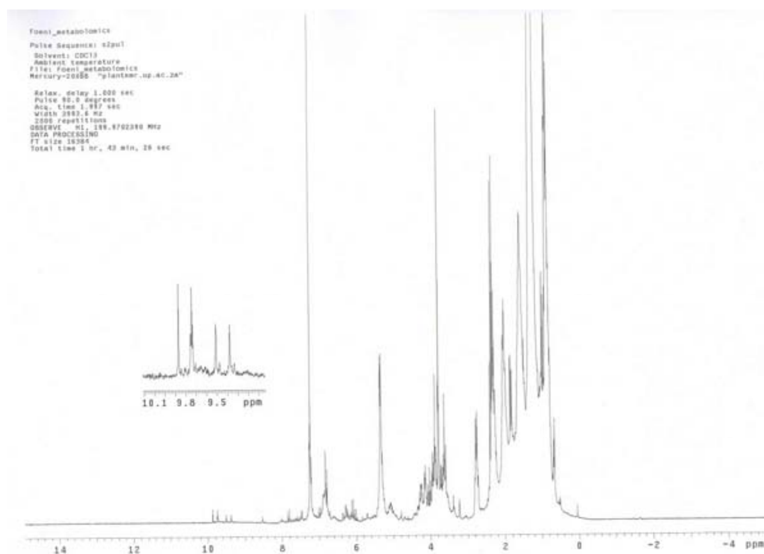


Fig. 3.7. ^{1}H -NMR spectrum of *Foeniculum vulgare*.

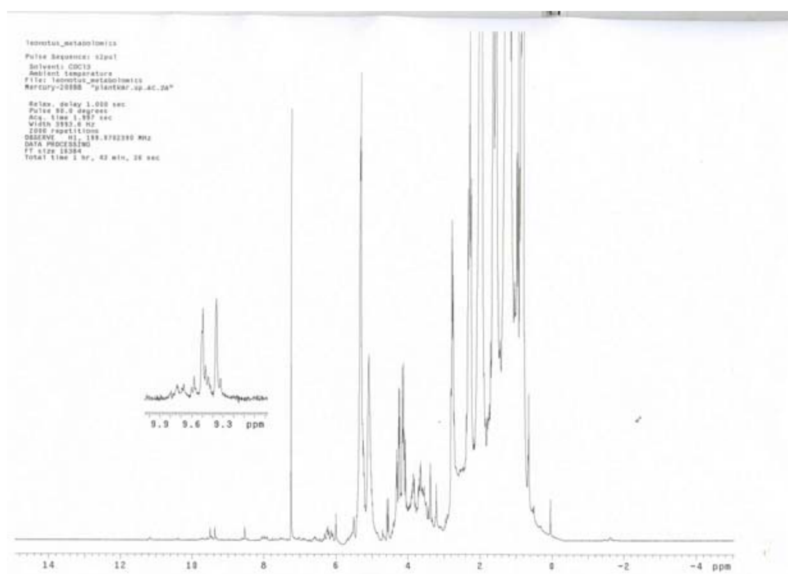


Fig. 3.8. ^{1}H -NMR spectrum of *Leonotis leonorus*.

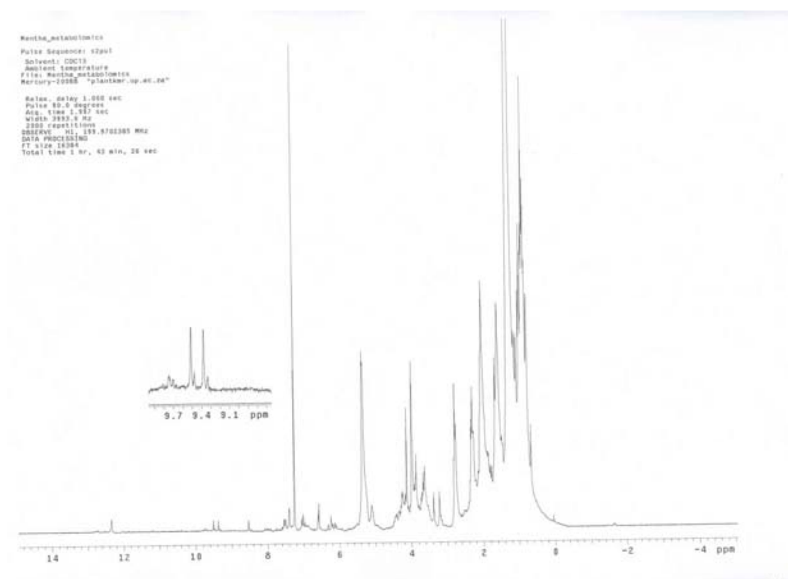


Fig. 3.9. ^{1}H -NMR spectrum of *Mentha longifolia.*

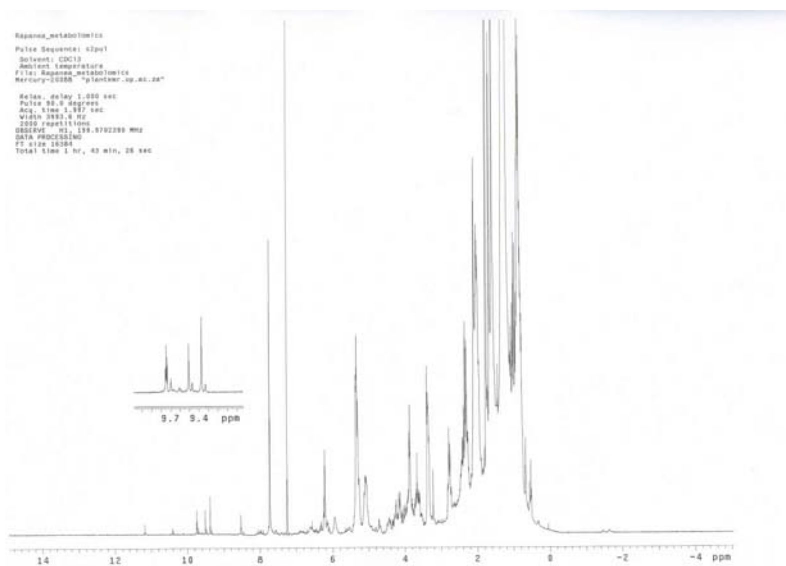


Fig. 3.10. ^{1}H -NMR spectrum of *Rapanea melanophloes*.

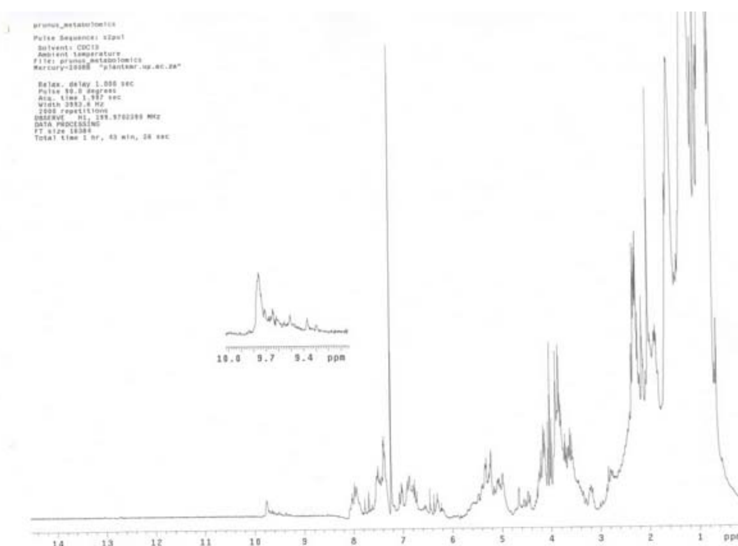


Fig. 3.11. ^{1}H -NMR spectrum of *Prunus africana.*

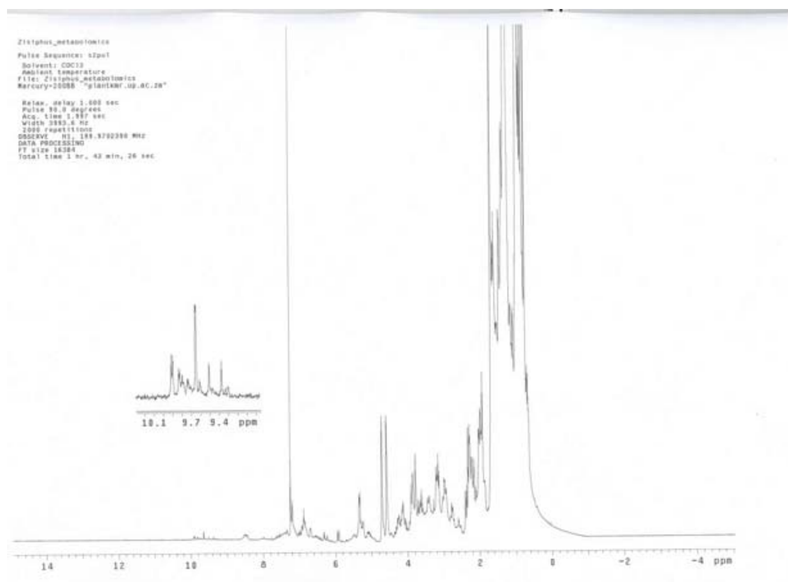


Fig. 3.12. ^{1}H -NMR spectrum of *Zisiphus mucronata.*

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.

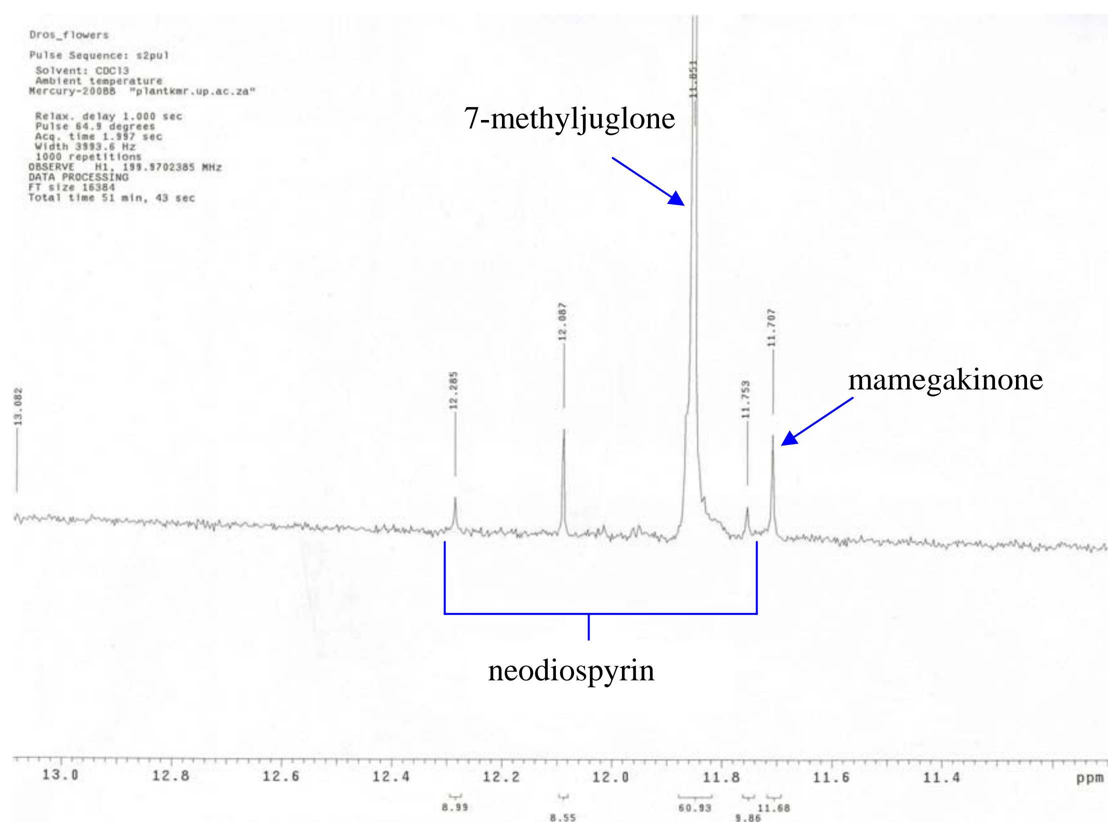


Fig. 3.13.The ^{1}H-NMR spectrum of the hydroxyl region confirming the identity of the compounds.

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

Caniato, R., Filippini, R. & Cappelletti, E. M. (1989). Naphthoquinone contents of cultivated *Drosera* species: *Drosera binata*, *D. binata* var. *dichotoma* and *D. capensis*. *International Journal of Crude Drug Research*. 27(3), 129-36.

Dialameh, G. H. (1978). Stereobiochemical aspects of warfarin isomers for inhibition of the enzymic alkylation of menaquinone -0 to menaquinone -4 in chick liver. International *Journal for Vitamin and Nutrition Research*. 48(2), 131-5.

Evans, C.C. (1998). Historical background. In: Clinical tuberculosis, ed. P.D.O. Davies, pp. 3,17. Chapman & Hall Medical, London.

Kapadia, N. S., Isarani, S. A. & Shah, M. B. (2005). A Simple Method for Isolation of Plumbagin from Roots of *Plumbago rosea*. *Pharmaceutical Biology (Philadelphia, PA, United States)*. 43(6), 551-553.

Lee, K. & Campbell, R.W. (1969). Nature and occurrence of juglone in *Juglans nigra*. *HortScience*. 4(4), 297-8.

Lillie, T. J. & Musgrave, O. C. (1977). Ebenaceae extractives. Part 7. Use of hydroxy-proton shifts of juglone derivatives in structure elucidation. *Journal of the Chemical Society, Perkin Transactions 1*: 355-359.

Mallavadhani, U.V., Panda, A.K. & Rao, Y.R. (1998). Pharmacology and chemotaxonomy of *Diospyros*. *Phytochemistry*. 49: 901-951.

Marczak, L., Kawiak, A., Lojkowska, E. & Stobiecki, M. (2005). Secondary metabolites in *in vitro* cultured plants of the genus *Drosera*. *Phytochemical Analysis*. 16(3), 143-149.

Suzuki, T., Haga, K., Kataoka, M., Tsutsumi, T., Nakano, Y., Matsuyama, S. & Kuwahara, Y (1995). Secretion of thrips. VIII. Secretions of the two *Ponticulothrips* species (Thysanoptera: Phlaeothripidae). *Applied Entomology and Zoology*. 30(4), 509-19.

Tokunaga, T., Dohmura, A., Takada, N. & Ueda, M. (2004). Cytotoxic antifeedant from *Dionaea muscipula* Ellis: a defensive mechanism of carnivorous plants against predators. *Bulletin of the Chemical Society of Japan*. 77(3), 537-541.

Van der Viyver, L.M. & Gerritsma, K.W. (1974). Naphthoquinones of *Euclea* and *Diospyros* species. *Phytochemistry*. 13, 2322-2323.

Van Wyk BE., Van Oudshoorn, B. & Gericke, N. (2002). Medicinal plants of South Africa, pp 110, 132, 290. Briza Publications, Arcadia, Pretoria.

Villas-Boas, S.G., Rasmussen, S. & Lane, G.A. (2005). Metabolomics or metabolite profiling. *Trends in biotechnology.* 23.(8) 385-386.

University of Pretoria etd – Van der Kooy, F (2007)

Chapter 4

Synthesis of 7-methyljuglone and diospyrin

4.1 Introduction 57

4.2 Materials and methods 58

4.2.1 Materials 58

4.2.2 Methods 59

4.2.2.1 Synthesis of 7-methyljuglone 59

4.2.2.1.1 Effect of different quantities of reagents on 7-methyljuglone formation 59

4.2.2.1.2 Effect of different quantities of catalyst on 7-methyljuglone formation 60

4.2.2.1.3 Influence of temperature on 7-methyljuglone formation 60

4.2.2.1.4 Effect of altering reaction times 61

4.2.2.2 Epoxidation of 7-methyljuglone 61

4.2.2.2.1 Effect of reaction time on epoxide formation 61

4.2.2.2.2 Influence of time before acidification 61

4.2.2.2.3 Effect of amount of acid on epoxide formation 62

4.2.2.3 Synthesis of diospyrin 62

4.2.2.3.1 Oxidative dimerisation of 7-methyljuglone **62**

4.2.2.3.2 Buffered reaction between hydroquinone and 7-methyljuglone **62**

4.2.2.3.3 Epoxide condensation **63**

4.2.2.3.3.1. Addition of an Bronsted-Lowry acid to the epoxide 63

4.2.2.3.3.2 Addition of an Lewis acid and steam distillation 63

4.3 Results 64

4.3.1 Synthesis of 7-methyljuglone 64

4.3.1.1 Effect of different quantities of reagents on 7-methyljuglone formation 64

4.3.1.2 Effect of different catalyst ratios 64

4.3.1.3 Influence of temperature on 7-methyljuglone formation 65

4.3.1.4 Effect of altering stirring times 65

4.3.2 **Epoxidation of 7-methyljuglone** **66**

4.3.2.1 **Influence of reaction time on epoxide formation** **66**

4.3.2.2 **Effect of time before acidification** **66**

4.3.2.3 **Effect of the amount of acid** **67**

4.3.3 **Synthesis of diospyrin** **68**

4.3.3.1 **Oxidative dimerisation of 7-methyljuglone** **68**

4.3.3.2 **Buffered reaction between hydroquinone and 7-methyljuglone** **68**

4.3.2.3 **Epoxide condensation** **68**

4.3.2.3.1 ***Addition of Bronsted-Lowry acid*** ***68***

4.3.2.3.2 ***Addition of Lewis acid and steam distillation*** ***69***

4.4 **Discussion and conclusions** **70**

4.5 **References** **74**

Chapter 4

Synthesis of 7-methyljuglone and diospyrin

4.1 Introduction

According to McMurry (1996) and Morrison & Boyd (1992), organic chemistry is the chemistry of carbon compounds. Although carbon is the principle atom in organic compounds, most compounds also contain hydrogen, nitrogen, oxygen or other elements. Carbon has the unique ability to bond together to form long chains and rings. Therefore carbon is able to form an immense diversity of compounds, from the simple (methane- one carbon atom) to the complex (DNA- billions of carbon atoms).

All living organisms produce organic compounds in order to be able to perform various metabolic processes. Plants do this primarily through their ability to photosynthesise. Animals use these products from plants to generate energy, grow and repair wounds. The physical and chemical properties of these compounds depend on the molecular structure of the compound. During previous studies a fairly simple organic compound, and its dimers, was isolated from *Euclea natalensis* A.DC. This compound, 7-methyljuglone**,** was first synthesized by Cooke & Dowd, (1952) and was re-investigated and improved by Musgrave & Skoyles (2001), in a two-step procedure. They used the Friedel-Crafts acylation reaction between maleic anhydride and 4-chloro-3-methylphenol to form 8-chloro-7-methyljuglone. This chlorinated product was then reduced during the second step with tin chloride to form 7-methyljuglone**.** Both steps in this synthetic pathway was low yielding and difficult to perform. The overall yield of the two-step procedure (3 % calculated from the publication) is not satisfactory. The compound was synthesized in a one-step procedure (also employing a Friedels-Craft acylation reaction) during a previous study (Van der Kooy, 2003). Although the one-step synthesis saved valuable time and costs, it only gave yields of less than 1 %. It was attempted to improve the yield in this part of the study (Van der Kooy & Meyer, 2006).

Only one publication could be found regarding the synthesis of diospyrin (Yoshida & Mori, 2000). This synthetic route entails a 14 step sequence with yields of less than 4 %. Keeping in mind the costs, time and the experience needed for such a synthesis it was decided that an alternative pathway was needed. Kumari *et al.* (1982) and Sankaram *et al.* (1975) published a

two-step pathway for the synthesis of neodiospyrin and a plumbagin dimer, respectively. Due to the structural similarities between neodiospyrin and the plumbagin dimer with diospyrin**,** this synthetic pathway was further investigated. In addition a new 3-step synthetic route with 7-methyljuglone epoxide as intermediate was investigated.

There are a couple of advantages in being able to synthesise a natural product:

- Under some circumstances synthesising the compound might be cheaper than isolating the compound. In the case of 7-methyljglone and some of its dimers this is almost certainly the case.
- Plant material does not have to be used, which will save plant resources from exploitation.
- Another advantage is the increase in yields. Theoretically any amount of compound can be synthesised according to the needs of future experiments.
- The synthetic compound also proves that the proposed structure of the isolated compound is correct. This advantage is of more use when complex compounds are worked with.

In this chapter the one-step synthetic pathway is revisited. Although the Friedel-Crafts reaction in this case is a low yielding procedure, the aim of any synthetic route should be to obtain reproducible and high yields. In addition to 7-methyljuglone, the chemical synthesis of diospyrin was also investigated with three different approaches.

4.2 Materials and methods

4.2.1 Materials

All chemicals used were of AR purity. The products of the different reactions were tested on silica gel TLC (hexane:ethyl acetate 5:2) and HPLC using phenomenex RP18 column (150 X 4.6 mm, 4 μ) with 62.5 % acetonitrile and 5 % acetic acid as mobile phase. A flow rate of 1 ml/min was employed with the detection system a UV6000LP PDA detector. The NMR analysis was done on a Varian Mercury-Plus 200 MHz with $CDCl_3$ as internal standard and referenced to 7.24 ppm.

4.2.2 Methods

4.2.2.1 Synthesis of 7-methyljuglone

To obtain the highest yielding method, the ratios between reagents and parameters such as stirring time and temperature of the reaction was tested. Different ratios of anhydrous aluminium chloride and sodium chloride were melted and different ratios of *m*-cresol and maleic anhydride was added into it. After addition, the mixture was vigorously stirred and added to a mixture of 12 N hydrochloric acid and crushed ice. The resulting precipitate was filtered off after 20 min, washed with water and dried. The crude brown material was extracted repeatedly with hexane. The different yields of 7-methyljuglone were calculated (from HPLC) and from this data the optimal conditions for this synthetic pathway were obtained.

4.2.2.1.1 Effect of different quantities of reagents on 7-methyljuglone formation

To establish the optimum ratio of the two starting reagents in the reaction, it was decided to use different quantities in molar equivalents. The reagents were added to $AlCl_3$ and NaCl at 180ºC. After 2 min of stirring it was added into 30 ml of HCl and crushed ice. After 20 min the mixture was filtered and washed with water. The reaction was repeated with different ratios and the appearance of the product noted. The products were extracted and tested on TLC for 7-methyluglone formation. Table 4.1 gives the different ratios that were used.

Table 4.1: Ratios of maleic anhydride and *m*-cresol tested to obtain the highest yield. Masses are in grams with molar equivalents in brackets (1 eq).

Maleic anhydride	*m*-Cresol	$AlCl_3$	NaCl
1.00g (1.00)	0.37g (0.30)	12.00g (8.80)	2.40g (4.10)
1.00g (1.00)	0.74g (0.60)	12.00g (8.80)	2.40g (4.10)
1.00g (1.00)	1.11g (1.00)	12.00g (8.80)	2.40g (4.10)
1.00g (1.00)	2.22g (2.00)	12.00g (8.80)	2.40g (4.10)

4.2.2.1.2 Effect of different quantities of catalyst on 7-methyljuglone formation

The optimum ratio of reagents was used (1.00 g of maleic anhydride and 1.11 g of m-cresol) and the catalyst ratios were changed. The same procedure was followed and the reaction repeated with the different ratios. The appearance of the melt was noted and the amount of 7-methyljuglone was established with the use of HPLC.

Table 4.2. Ratio of catalyst that was used to obtain the highest yields

$AlCl_3$ (grams)	NaCl (grams)	Appearance	Temperature (°C)
12.0	0.0	Does not melt	160
12.0	1.2	Dark brown	180
12.0	2.4	Light brown	180
12.0	3.6	Turns sticky after 1.5 min of stirring	180

4.2.2.1.3 Influence of temperature on 7-methyljuglone formation

The effect that the reaction temperature had on 7-methyljuglone formation was tested by using the optimum amount of reagents and catalyst and by changing the temperature during three separate reactions. The temperatures used and the appearance of the crude product is given in Table 4.3.

Table 4.3: Effect of different temperatures on 7-methyljuglone formation. The optimum ratio of maleic anhydride and m-cresol were used

Maleic anhydride (grams)	m-cresol (grams)	$AlCl_3$ (grams)	NaCl (grams)	Temp °C	Appearance
1.00	1.11	12.00	2.40	200	Dark brown
1.00	1.11	12.00	2.40	175	Light brown
1.00	1.11	12.00	2.40	150	Yellow

4.2.2.1.4 Effect of altering reaction times

The optimum conditions were used and the reaction times changed. The reaction was repeated and the mixture stirred for 30 s, 1 min, 2 min and 3 min after which it was added into HCl (30 ml) and crushed ice. The products were filtered and extracted with hexane after which it was tested on HPLC.

4.2.2.2. Epoxidation of 7-methyljuglone

Twenty five mg of 7-methyljuglone was dissolved in 10 ml of ethanol at 0 ºC. To this mixture a mixture of sodium perborate (62.5 mg) in water (7 ml) was added. After 2 min the pH was adjusted to 5 with 2 M HCl. A saturated mixture of NaCl in water (5 ml at 0 ºC) was added and the mixture extracted with hexane after 4 min. The crude product was tested on HPLC and NMR.

4.2.2.2.1 Effect of reaction time on epoxide formation

It was noted that two dimers also appeared during the epoxidation reaction. One of these had a similar retention time on the HPLC than diospyrin**.** This compound might have formed due to the reaction time or the over acidification of the extract. The analysis was therefore repeated and the solution added to hexane at different time intervals. Ten mg of 7-methyljuglone were dissolved in 4 ml EtOH at 0 ºC. To this mixture sodium perborate (25 mg) in water (2.8 ml) was added. The pH was adjusted with 2 M HCL to pH 5 after 2 min, after which 2 ml of NaCl was added. The reaction was continued in separate experiments for 1, 4, 10, 20 and 60 min. Extracting the products after the different time intervals with hexane stopped the reactions, after which it was tested on HPLC.

4.2.2.2.2 Influence of time before acidification

The reaction was repeated as described above with the exception that the pH was adjusted after 30 s, 60 s, 120 s, 240 s and 300 s (5 drops HCl) in separate experiments. After the addition of acid the NaCl solution was added and the products extracted after 4 min. The products were tested on HPLC for the formation of the dimeric forms and the conversion into the epoxide.

4.2.2.2.3 Effect of amount of acid on epoxide formation

The same reaction was repeated with the exception that the amount of acid used varied. The reaction mixture was added to different volumes of acid after 4 min. The following quantities were used: no acid, 5 drops, 10 drops, 20 drops. After the addition of the acid, 4 ml NaCl was added.

4.2.2.3 Synthesis of Diospyrin

The synthesis of diospyrin has been achieved recently by Yoshida & Mori (2000). The synthesis entails 14 steps with very low overall yields which makes this method not commercially viable. There are however a couple of ways to dimerise a compound of this nature. Van der Vjiver & Gerritsma (1974) reported that neodiospyrin and mamegakinone forms through oxidative dimerisation when 7-methyljuglone is exposed to air in a silica gel matrix. This method was investigated. The synthesis of neodiospyrin in a phosphate buffer, which was achieved in previous studies, was also reinvestigated by changing the pH of the buffer. The aim was to stop the formation of neodiospyrin and to synthesise diospyrin**.** A third reaction was investigated through which 7-methyljuglone was epoxidised and allowed to react with itself with the loss of water.

4.2.2.3.1 Oxidative dimerisation of 7-methyljuglone

Synthetic 7-methyljuglone (5 mg) was added to 1 g of silica gel. The mixture was stirred and air was passed over it for 6 weeks. Samples were collected at weekly intervals by dissolving 100 mg of the mixture in chloroform and filtering the silica off. The filtrate was injected into the HPLC with an authentic diospyrin standard.

4.2.2.3.2 Buffered reaction between 7-methyljuglone and its hydroquinone

7-Methyljuglone (25 mg) was dissolved in 2.5 ml of tetrahydrofuran (THF). This was added to a solution of tin chloride (125 mg), 2.5 ml of THF and 8.75 ml of 4 M hydrochloric acid (HCl) at 60 ºC. The solution was stirred for 3 hours. After stirring, the THF was evaporated by opening the vessels for 15 min at 60 ºC, after which the THF was completely evaporated

with the use of a rotary evaporator. The reduced 7-methyljuglone was recovered by washing out the excess HCl and tin chloride in a reverse phased silica gel column (solid phase extraction). The column was prepared by conditioning it with methanol (MeOH) and then with water. The sample was introduced and the HCl and tin chloride removed by washing the column with water. The reduced 7-methyljuglone remained in the silica column and was collected by washing the column with MeOH.

A phosphate buffer was prepared by dissolving 6.8 g of KH_2PO_4 in 1 L of water. The pH was adjusted separately to 4.6, 5.6, 6.6, 7.6 and 8.6 with 3 % KOH. Unreduced 7-methyluglone (25 mg) was dissolved in the minimum amount of MeOH and 25 ml of phosphate buffer. The solution was stirred while adding the reduced 7-methyljuglone (hydroquinone), dissolved in the minimum amount of MeOH, into the reaction vessel. The solution was stirred for 30 min. The resulting precipitate that formed was filtered off, washed with water and dried. This crude product was tested on HPLC for purity with an authentic diospyrin and neodiospyrin standard.

4.2.2.3.3 Epoxide condensation

4.2.2.3.3.1. Addition of an Bronsted-Lowry acid to the epoxide

The 7-methyljuglone epoxide was dissolved in an acetone / water mixture and the acetone evaporated. A couple of drops of 4 M HCl were added to the water mixture. The reaction was heated to 60 ºC for 1 hour after which a brown solid precipitate appeared. The solid precipitate was filtered off and tested on NMR and HPLC.

4.2.2.3.3.2. Addition of an Lewis acid and steam distillation

The 7-methyljuglone epoxide (10 mg) was dissolved in the 20 ml of THF. This was added to a round bottom flask containing 56 mg of SnCl2 in 20 ml of water. The mixture was heated to 60 °C without vacuum on a rotary evaporator. The mixture was tested on HPLC after 1 min. 30 min and 2 hours. After 2 hours the vacuum was switched on and the distillate (yellow colour) and reaction mixture were tested on HPLC. The reaction was continued for 2 days without vacuum and THF was added when solid material appeared. After 2 days, 10 mg of 7-methyljglone in THF was added to the reaction mixture. The mixture was again distilled and tested on HPLC.

4.3 Results

4.3.1 Synthesis of 7-methyljuglone

4.3.1.1 Effect of different quantities of reagents on 7-methyljuglone formation

The products of the various reactions were tested on TLC plates and on HPLC with an authentic 7-methyljuglone standard. The different ratios of reactants gave the best result when a 1:1 molar equivalent was used. This translates into using 1.0 g of maleic anhydride and 1.11 g of *m*-cresol. With the other reactions none or very small amounts of 7-methyljuglone was obtained. The appearance of more by-products was also seen when the other ratios were used.

4.3.1.2 Effect of different catalyst ratios

The catalyst quantities were tested at four different ratios. During the first analysis no salt was used. The $AlCl_3$ did however not melt and the temperature stayed at 160 ºC. Therefore the salt was needed to assist in the melting of $AlCl_3$. It was previously thought that the salt might play a role in the reaction itself. Table 4.4 gives the ratios used with the resulting 7-methyljuglone formation.

.

Table 4.4: Ratio of catalyst used

$AlCl_3$ (grams)	NaCl (grams)	Appearance	Reaction temperature (ºC)	Presence of 7-methyljuglone
12.0	0.0	Does not melt	160	No
12.0	1.2	Sticky darker brown	180	Yes
12.0	2.4	Brown	180	Yes
12.0	3.6	Turns sticky-solid after 1.5 min	180	Yes

The ratio of 12 g $AlCl_3$ and 2.4 g of NaCl appeared to give 7-methyljuglone in the largest amount. The appearance of the amount of by-products was also noted in each reaction.

4.2.1.3 Influence of temperature on 7-methyljuglone formation

The reaction was repeated with the optimum ratios of reactants and catalyst at 3 different temperatures. The presence of 7-methyljuglone and any by-products were tested on TLC. The appearance of the crude product was also indicative of the amount of 7-methyljuglone present. The reaction at 200 ºC gave the best results while at 150 ºC no 7-methyljuglone formed (Table 4.5).

Table 4.5: Effect of different temperatures on 7-methyljuglone formation. The optimum ratio of maleic anhydride and m-cresol were used

Temperature (ºC)	Appearance	Presence of 7-methyljuglone
200	Dark brown	Yes –very little by-products
175	Light brown	Yes – more by-products
150	Yellow	No

4.1.2.4 Effect of altering stirring times

The time of the reaction was also tested. After addition of the reactants to the molten catalyst the mixture was stirred for 30 s, 60 s, 120 s and 240 s after which it was added into HCl and ice. The appearance of the product and the amount of catalyst was noted. Stirring time of 60-120 s gave the best results. Longer stirring time resulted in a very sticky mixture with lower amounts of 7-methyljuglone.

The reaction was repeated with all the optimum conditions (1 g maleic anhydride, 1.11 g m-cresol, 12 g $AlCl_3$, 2.4 g NaCl, at 200 ºC with stirring time of 120 s). The resulting precipitate was filtered and washed with water. After drying the solid material it was extracted with hexane. A silica column was prepared with hexane ethyl acetate 9:1 as eluent. The 7-methyljuglone fractions were collected and a ^{1}H-NMR was performed. Fig 4.1 gives the proton NMR spectrum.

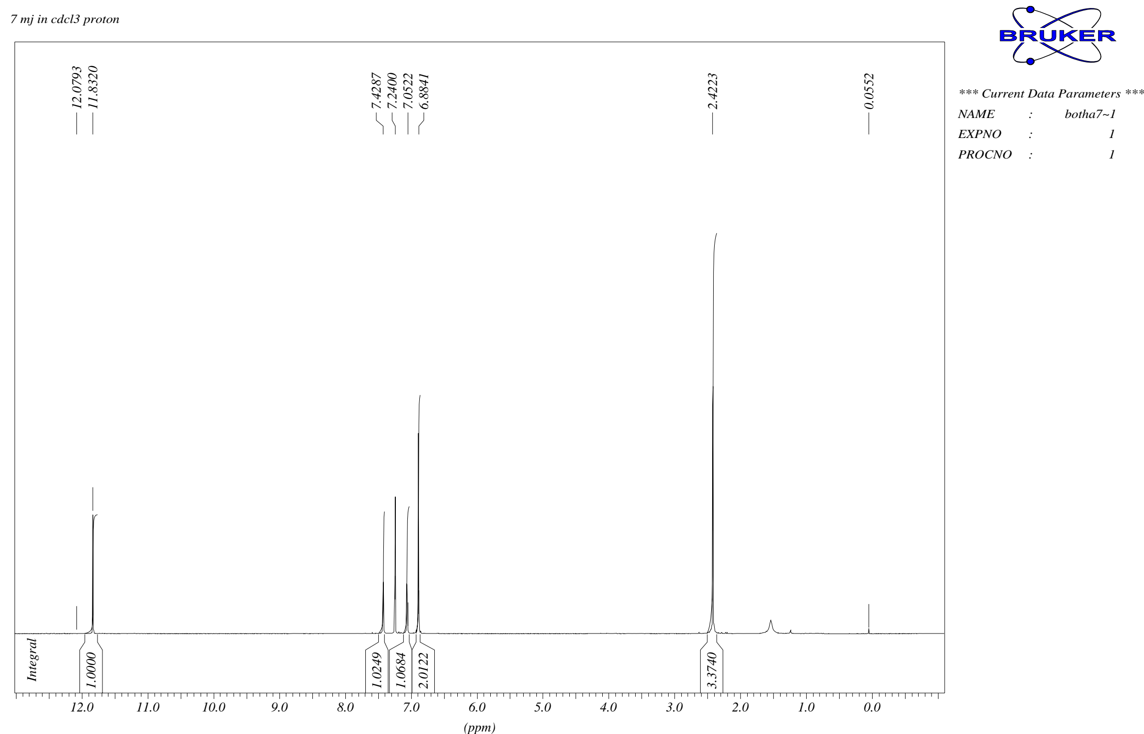


Fig. 4.1: ^{1}H-NMR spectrum of the synthetic 7-methyljuglone**.**

4.3.2 Epoxidation of 7-methyljuglone

The epoxidation of 7-methyljuglone yielded approximately a 60:40 ratio of the epoxide to the unreacted 7-methyljuglone as tested on HPLC. It was therefore necessary to improve this method.

4.3.2.1 Influence of reaction time on epoxide formation

The analysis was repeated and the solution stopped by adding it to hexane at five different time intervals. The resulting products were tested on HPLC. No difference in the epoxide formation was noted. The two dimers that formed were also not influenced by the reaction time. The addition of the acid therefore effectively stopped the reaction.

4.3.2.2 Effect of time before acidification

The addition of acid to the reaction mixture was tested at different time intervals. At 240 and 300 s the 7-methyljuglone was completely converted into its epoxide. It did not however influence the formation of the dimeric forms.

4.3.2.3 Effect of the amount of acid

The reaction was repeated and it was decided to change the amount of acid that was added. Five different volumes of acid were added and the resulting products were tested on HPLC. The epoxide itself did form in all the cases, but no difference was noted in the dimer formation. The crude products were tested on NMR to identify the dimers. The hydroxy proton shifts of mamegakinone and diospyrin could be seen (Fig. 4.2). It is therefore a by-product of the epoxidation step. It was however noted that the presence of 7-methyljuglone was needed before the dimers formed. It is therefore plausible that the epoxide reacts with 7-methyljuglone to give diospyrin and mamegakinone.

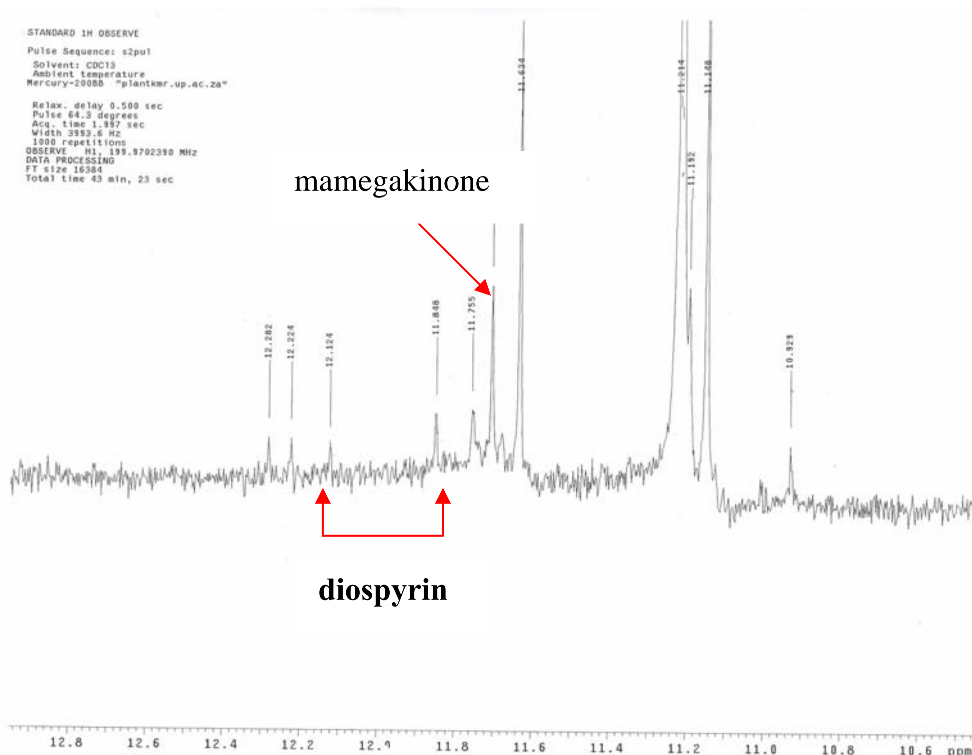


Fig 4.2 Hydroxy proton shifts, on the ^{1}H-NMR spectrum, indicating the presence of diospyrin and mamegakinone

4.3.3 Synthesis of diospyrin

4.3.2.1 Oxidative dimerisation

After aerating the sample in silica gel for 6 weeks it was tested on HPLC. The sample did appear to undergo some major changes. A very small amount of 7-methyljuglone was still present but a lot of other compounds did form. None of these compounds was diospyrin.

4.3.2.2 Buffered reaction between hydroquinone and 7-methyljuglone

The colour of the reactions was important (Table 4.5). A purple colour indicated the appearance of the 7-methyljuglone potassium salt. This appears to be a crucial step in the formation of the dimers. Neodiospyrin did form, but there was no trace of diospyrin. The atomic radius of the potassium atom (227 pm) is large compared to the rest of the molecule. It might therefore be possible that the carbon 6 atom is sterically hindered to take part in the reaction. Future experiments should therefore make use of smaller atoms (Na = 186 pm, Li = 152 pm).

Table 4.5: The formation of the dimer, neodiospyrin at different pH's. The colour of the reaction was also noted.

pH	4.6	5.6	6.6	7.6	8.6
Reaction colour	Orange	Green-brown	Dark purple – brown	Purple-brown green	Purple -green
Dimer formation	no	no	Yes - neodiospyrin	Yes - neodiospyrin	no

4.3.2.3 Epoxide condensation

4.3.2.3.1 Addition of Bronsted-Lowry acid

The epoxide reacted rapidly with HCl to form the expected 2-chloro-3-hydroxy-7-methyljuglone. NMR analysis done on the crude product indicated that this was the major product. No dimers occurred during any stage of this reaction. The use of HCl will unfortunately always give the chlorinated product. It was therefore decided that a Lewis acid should be used.

4.3.2.3.2 Addition of Lewis acid and steam distillation

After 2 days the formation of a small amount of diospyrin in the reaction vessel was noted. The distillate contained 7-methyljuglone, 7-methyluglone epoxide and the reduced epoxide. The reaction vessel contained small amounts of diospyrin. The major constituents of the vessel were still the epoxide and 7-methyljuglone. The sample was tested on HPLC and it appeared to be diospyrin. An authentic standard of diospyrin was injected with the sample and only one well-resolved peak at the correct retention time was noted. The sample was subjected to NMR and small hydroxy proton peaks at the correct chemical shifts (Lillie & Musgrave, 1977) were noted. The signal to noise ratio was very low and therefore the NMR analysis can not be seen as conclusive proof of diospyrin formation. Fig 4.3 gives the HPLC chromatogram indicating the presence of diospyrin at rt 9.496 min.

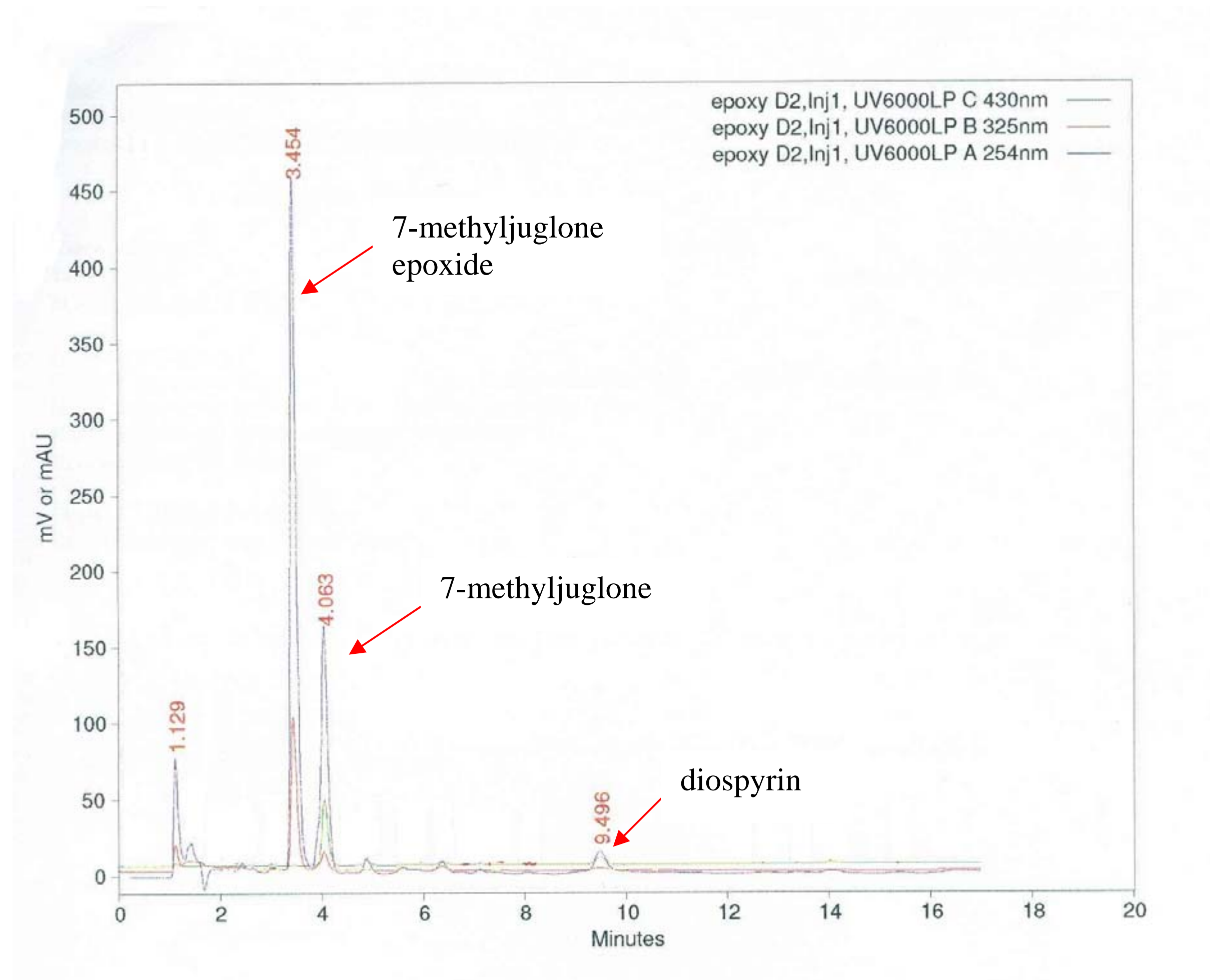


Fig. 4.3: The presence of a small amount of diospyrin, on the HPLC chromatogram, can be seen at a retention time of 9.496 min.

4.4 Discussion and conclusions

The synthesis of 7-methyljuglone remains a low yielding method (16 %). It was however improved in such a way that the one-step synthesis can be used instead of the published two-step synthesis. With the variation in the amount of starting reagents used, it was quite clear that a 1:1 molar equivalent gave the best yields. The catalyst with the salt also gave a clear indication that the salt is needed in order to melt the mixture. It therefore plays an important part in the viscosity of the mixture. The previously used temperature of 180 ºC appears to be correct. It might be significant that temperatures above this could dramatically increase the yields. We did however not attempt any temperatures in excess of 200 ºC due to safety concerns (The flash points of the different chemicals were taken into account in this decision). In future a reactor can be used which can be operated under reduced pressure and thereby higher temperatures. The effect of the stirring time on the reaction indicated that the optimum time was between 1 and 2 minutes. The purification step proves to be considerably more difficult as the *m*-cresol (unreacted) and 7-methyljuglone overlaps in the current column system that was used. This can however be improved on with the use of liquid - liquid separation or a different mobile phase in the column system. This was however not the purpose of the experiment.

The reaction mechanism is not yet fully understood. Fig. 4.4 illustrates a probable mechanism. The mechanism for the reaction involves either the formation of an acylium ion as the reactive electrophilic species, but it might also involve an electrophilic complex between the acid anhydride and the aluminium chloride (Hanneford *et al*., 1989). We propose that an electrophilic complex forms between the catalyst and the ketone group. This creates ring opening with nucleophilic attack in the *ortho* position from the aromatic ring to the partially positively charged carbon. Lower temperatures favours attack from the *para* position (Benson *et al*. 2001). A second molecule of $AlCl_3$ forms a complex with the second ketone group as the process is repeated. This reaction sequence probably occurs in a single step with two $AlCl_3$ molecules reacting at once with the anhydride. The end result is 7-methyljuglone with the loss of one molecule of water.

Diospyrin synthesis via oxidative dimerisation did not yield the correct results. No diospyrin formed after 6 weeks even when the experiment was repeated with added $FeCl_3$ as catalyst. There are still some variations that could be tried but it was abandoned in favour of the

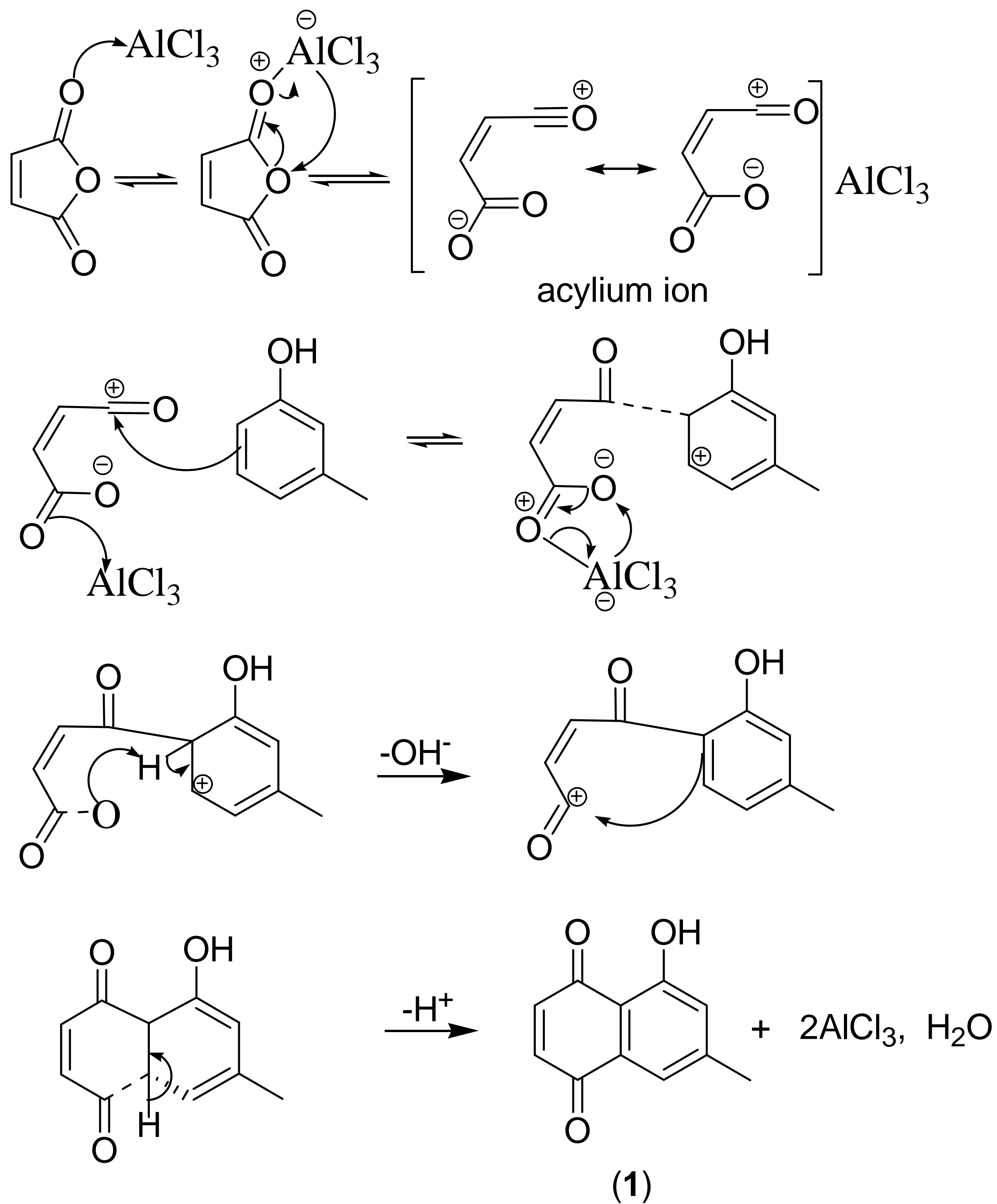


Fig. 4.4: Proposed reaction mechanism for 7-methyljuglone synthesis

epoxide and reductive coupling of 7-methyljuglone in buffer reactions. The reaction time becomes important when the synthesis is commercialised.

The buffer reaction still shows promise and it should be repeated with the use of different bases. The pH can also be adjusted in smaller steps. In future a lithium or sodium base should be used in the buffer and the pH should be changed in smaller steps from pH 5 to pH 7. The occurrence of neodiospyrin in high yields should make it possible to alter conditions so that diospyrin forms instead.

The epoxidation of 7-methyljuglone and the improvement of the method led to the occurrence of two dimers. The one dimer could positively be identified as mamegakinone (due to its hydroxy proton shift). Unfortunately the hydroxy proton shift of one hydroxyl ion of diospyrin overlaps with approximately 4 other similar compounds. The other resonance peak was clearly present. Fig. 4.5 illustrates the mechanism of diospyrin formation. In theory it is possible to reduce an epoxide group to a double bond with the use of steam distillation. This process eliminates one molecule of water.

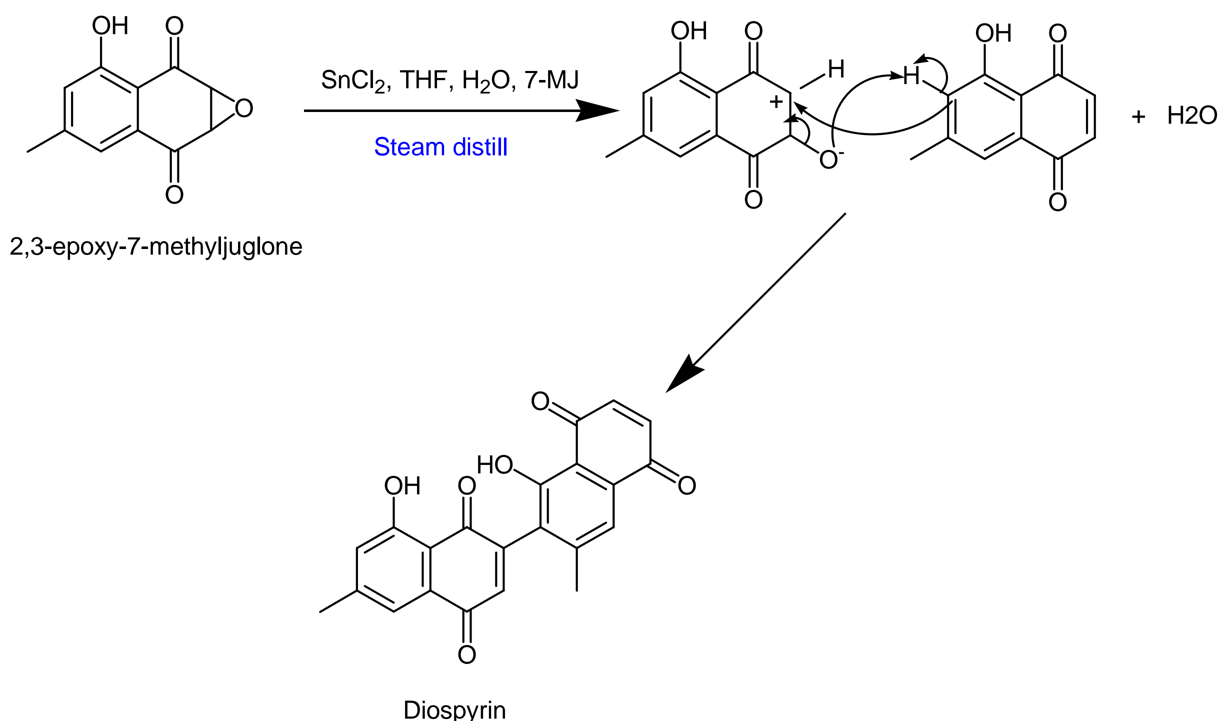


Fig. 4.5: Possible mechanism for diospyrin formation

4.5 References

Hanneford, A.J., Smith P.W. & Tatchell, A.R. eds. (1989). Vogel's Textbook of Practical Organic Chemistry 5th ed., Longman scientific & Technical, Essex, United Kingdom, pp. 1006-1007.

Benson, G.A., Maughan, P.J., Shelley, D.P. & Spillane, W.J. (2001). A new synthetic route to arylhydroxysulfonamides via a novel Fries-type rearrangement of aryl N,N-dialkylsulfamates. *Tetrahedron letters*. 42, 8729-8731.

Cooke, R.G. & Dowd, H. (1952). Colouring matters of Australian plants. III. Synthesis of 7-methyljuglone and related compounds. *Australian Journal of Chemistry.* 1: 53-57.

Kumari, L.K., Babu, M.H. & Pardhasaradhi, M. (1982). Synthesis of neodiospyrin & fixation of aryl-quinone linkage in its structure. *Indian Journal of Chemistry.* 21B: 619-621.

McMurry, J. (1996). Organic chemistry. 4th ed. pp 1-3, 583, 882. Brookes/Cole Publishing, USA.

Morrison, R. T. & Boyd, R. N. (1992). Organic chemistry. 6th ed. pp 1-3, 666, 901, 764, 905. Prentice Hall International, Inc.

Lillie, T. J. & Musgrave, O. C. (1977). Ebenaceae extractives. Part 7. Use of hydroxy-proton shifts of juglone derivatives in structure elucidation. *Journal of the Chemical Society, Perkin Transactions 1*: 355-359.

Musgrave, O.C. & Skoyles, D. (2001). Ebenaceae extractives. Part11. The synthesis of 7-methyljuglone. A re-examination. *Journal of the Chemical Society. Perkin Transactions*. 1 : 1318-1320.

Sankaram, A.V.B., Rao, A.S. & Sidhu, G.S. (1975). Synthesis of naturally occurring binaphthoquinones and related compounds. A novel reaction between plumbagin and its hydroquinone. *Tetrahedron Letters*. 42: 3627-3630.

Van der Kooy, F. (2003). Characterisation, synthesis and antimycobacterial activity of naphthoquinones isolated from *Euclea natalensis.* Unpublished. M.Sc. dissertation. University of Pretoria. South Africa.

Van der Kooy, F. & Meyer, J.J.M. (2006). Synthesis of the antimycobacterial naphthoquinone, 7-methyljuglone and its dimer, neodiospyrin. *South African Journal of Chemistry*. 59: 60-61.

Van der Viyver, L.M. & Gerritsma, K.W. (1974). Naphthoquinones of *Euclea* and *Diospyros* species. *Phytochemistry*. 13: 2322-2323.

Yoshida, M. & Mori, K. (2000). Synthesis of diospyrin, a potential agent against Leishmaniasis and related parasitic protozoan diseases. *European Journal of Organic Chemistry.* 1313-1317.

University of Pretoria etd – Van der Kooy, F (2007)

University of Pretoria etd – Van der Kooy, F (2007)

Chapter 5

Stability of naphthoquinones

5.1	**Introduction**	**76**
5.2	**Materials and methods**	**77**
5.2.1	**Materials**	**77**
5.2.2	**Methods**	**77**
5.2.2.1	**Stability in dimethylsulfoxide**	**77**
5.2.2.2	**Stability in BACTEC buffer solution**	**78**
5.2.2.3	**Stability in vero cell toxicity bioassay buffer**	**78**
5.2.2.4	**Stability in 20 % DMSO/Agar mixture**	**78**
5.2.2.5	**Stability in broth used for mode of action studies**	**78**
5.3	**Results**	**79**
5.3.1	**Stability in dimethylsulfoxide**	**79**
5.3.2	**Stability in BACTEC buffer solution**	**82**
5.3.3	**Stability in vero cell toxicity bioassay buffer**	**83**
5.3.4	**Stability in 20 % DMSO/Agar mixture**	**84**
5.3.5	**Stability in broth used for mode of action studies**	**84**
5.4	**Discussion and conclusions**	**84**
5.5	**References**	**87**

University of Pretoria etd – Van der Kooy, F (2007)

Chapter 5

Stability of naphthoquinones

5.1. Introduction

The often overlooked chemical analysis that bioactive compounds should be subjected to, is their stability in solvents. Most published bioassays are accepted by scientists and the methodology can therefore be used to screen any new bioactive compound that has been found. Unfortunately these new bioactive compounds do not always remain stable during these bioassays. If it is found that the compounds are not stable, it is necessary to adapt bioassays, or change the molecules structure to be able to get an accurate result.

The various tests that have to be performed to investigate the naphthoquinones (NQ's) as potential TB drugs, makes use of several solvents. During this chapter the stability of predominantly two NQ's were tested. The NQ's, diospyrin and 7-methyljuglone, showed the best activity against TB in the BACTEC radiometric assay (Jenkins, 1998). In this assay, liquid medium is used and the growth of *Mycobacterium tuberculosis* is monitored radiometrically with the results available within 5-6 days. Only 7-methyljuglone were tested for stability in the BACTEC vials due to the radioactive nature of the ^{14}C labelled palmitic acid. The two NQ's, diospyrin and 7-methyljuglone, were also the only ones tested in mice due to the high costs of the experiment involved, while three other NQ's, juglone, menadione and plumbagin, were also included in some of the experiments listed below. The NQ's were tested in five different "solvents", namely.

a) Dimethylsulfoxide (DMSO)
b) BACTEC buffer (9H11 Middlebrook agar with OADC growth supplement)
c) Minimum essential medium (vero cell toxicity bioassay)
d) 20 % DMSO/ 7H9 broth/glycerol (*in vitro* mice work)
e) 7H9 Middlebrook broth that was used for mode of action studies.

The organic solvent DMSO was tested because it is the most commonly used solvent to dissolve hydrophobic compounds (Lipinski, 2004). It was used to dissolve the compounds and to prepare a

University of Pretoria etd – Van der Kooy, F (2007)

stock solution which was also sometimes stored for future use. The final concentration of DMSO is usually between 0.5 and 2 % in the various bioassays before it became toxic to the test organisms (Soerbye *et al*., 1993; Crawford & Braunwald, 1991). These concentrations of DMSO were used in the BACTEC and vero cell toxicity bioassays. The solutions were prepared and tested on HPLC. The stability of 7-methyljuglone in DMSO was also tested in a kinetic NMR experiment. For the *in vitro* mice work the compounds were dissolved in a mixture of DMSO and added to 7H9 Middlebrook broth in 5 % glycerol. The final concentration of the DMSO was 20 %. This mixture was also tested for stability on HPLC. All the tests were carried out in exactly the same way that they were performed during the actual bioassays.

5.2. Materials and methods

5.2.1 Materials

The BACTEC vials were obtained from Becton, Dickenson and Company, USA, while the 7H9 Middlebrook broth, OADC growth supplement, DMSO and glycerol from Sigma-Aldrich. The samples were injected into the HPLC with mobile phase 62 % acetonitrile and 5 % aqueous acetic acid. The column was a RP18 Phenomenex 150X4.6 mm, 5 μ. The PDA detector was set to 254, 325 and 430 nm.

5.2.2 Methods

5.2.2.1 Stability in dimethylsulfoxide

The five napthoquinones: diospyrin, juglone, menadione, 7-methyljuglone and plumbagin were dissolved in AR grade DMSO to a 1 mg/ml concentration. These solutions were injected into the HPLC every hour for the first 4 hours after dissolving and then daily for one week and weekly for 1 month. This experiment was repeated to confirm the results. An additional experiment was performed in duplicate, but at a concentration of 10 mg/ml for diospyrin and 7-methyljuglone, as this is the stock concentration for most bioassays used. The samples were sonicated for 30 sec before each injection. In addition, 5 mg of pure 7-methyljuglone was dissolved in deteurated DMSO and analysed in a kinetic study on a 200 MHz Varian NMR spectrometer. The spectra were acquired 1 hour apart for 6 hours.

University of Pretoria etd – Van der Kooy, F (2007)

University of Pretoria etd – Van der Kooy, F (2007)

5.2.2.2 Stability in BACTEC buffer solution

7-methyljuglone was dissolved in DMSO and injected into the BACTEC vial so that the final concentration was 100 µg/ml and 1 % DMSO. Due to cost constraints and the radioactive nature (contains ^{14}C-labelled palmitic acid) of the test, only 7-methyljuglone was tested. The vials were placed in an incubator at 37 ˚C in the dark and 20 µl was injected into the HPLC after addition of the compound. The test was performed in duplicate. The test was repeated every 30 min for the first 3 hours. As control only the BACTEC solvent with 1 % DMSO was injected without any 7-methyljuglone**.**

5.2.2.3 Stability in vero cell toxicity bioassay buffer

The five napthoquinones: diospyrin, juglone, menadione, 7-methyljuglone and plumbagin were dissolved in DMSO at 20 mg/ml and immediately diluted 200 times in complete Minimum Essential Medium (MEM). As control the solvent mixture was prepared without any addition of the compounds. The samples (at a final concentration of 100 µg/ml) was injected into the HPLC every 30 min for the first hour and then daily for two days. During the toxicity bioassay the stock solution was further diluted to the required concentrations. Due to the low concentration of 7-methyljuglone a stock solution of 100 µg/ml was prepared in order to achieve a good signal to noise ratio on the HPLC.

5.2.2.4 Stability in 20% DMSO/ 7H9 broth/glycerol

For the *in vivo* mice work, the compounds were dissolved in DMSO and added to 7H9 broth in 5 % glycerol to reach a final concentration of 20 % DMSO. The final concentration of diospyrin and 7-methyljuglone was 2.5 mg/ml. The standards were prepared and injected into the HPLC over a period of four days. The solutions were stored in the dark in the cold room at 4 ˚C. The test was repeated to confirm the results.

5.2.2.5 Stability in broth used for mode of action studies

7H9 broth was prepared by dissolving 2.45 g in 450 ml of distilled water. To this mixture 2 ml of glycerol was added. The solution was autoclaved and allowed to cool down to room temperature

University of Pretoria etd – Van der Kooy, F (2007)

before 50 ml of OADC growth supplement was added. The five naphthoquinones: diospyrin, juglone, menadione, 7-methyljuglone and plumbagin was dissolved in DMSO and added to the broth solution to a final DMSO concentration of 0.5 %. The stability was determined on HPLC over a period of three days. The blank sample contained only the broth with 0.5 % DMSO.

5.3 Results

5.3.1 Stability in dimethylsulfoxide

The stability of the NQ's were tested over a period of 1 month. Only menadione and plumbagin remained stable over that period. The most unstable compound appeared to be 7-methyljuglone**.** It started to break down after 30 minutes and after 1 month no traces of 7-methyljuglone could be found. Table 5.1 gives the rate of break down for the different NQ's. Fig. 5.1-5.4 contain HPLC chromatograms and the kinetic NMR spectrum of the breakdown of 7-methyljuglone in d-DMSO. The break down rate were higher at higher concentrations. Interestingly, the breakdown product were different at the different concentrations. The major product at 1 mg/ml appeared to be neodiospyrin, a dimeric form of 7-methyljuglone**.** At 10 mg/ml the major product was mamegakinone. Dimerisation therefore occurs in DMSO.

Table 5.1: The breakdown of the NQ's in DMSO over time displayed as average percentage remaining compound, as determined by HPLC.

Compound	3min	30min	1 day	1week
diospyrin	100.0	100.0 ± 0.4	68.9 ± 1.8	<5.0
juglone	100.0	100.0 ± 0.5	12.1 ± 2.8	<5.0
menadione	100.0	100.0 ± 0.3	100.0 ± 0.1	100.0 ± 0.3
7-methyljuglone	100.0	75.0 ± 2.4	7.5 ± 1.2	<5.0
plumbagin	100.0	100.0 ± 0.8	100.0 ± 0.4	100.0 ± 0.5

University of Pretoria etd – Van der Kooy, F (2007)

University of Pretoria etd – Van der Kooy, F (2007)

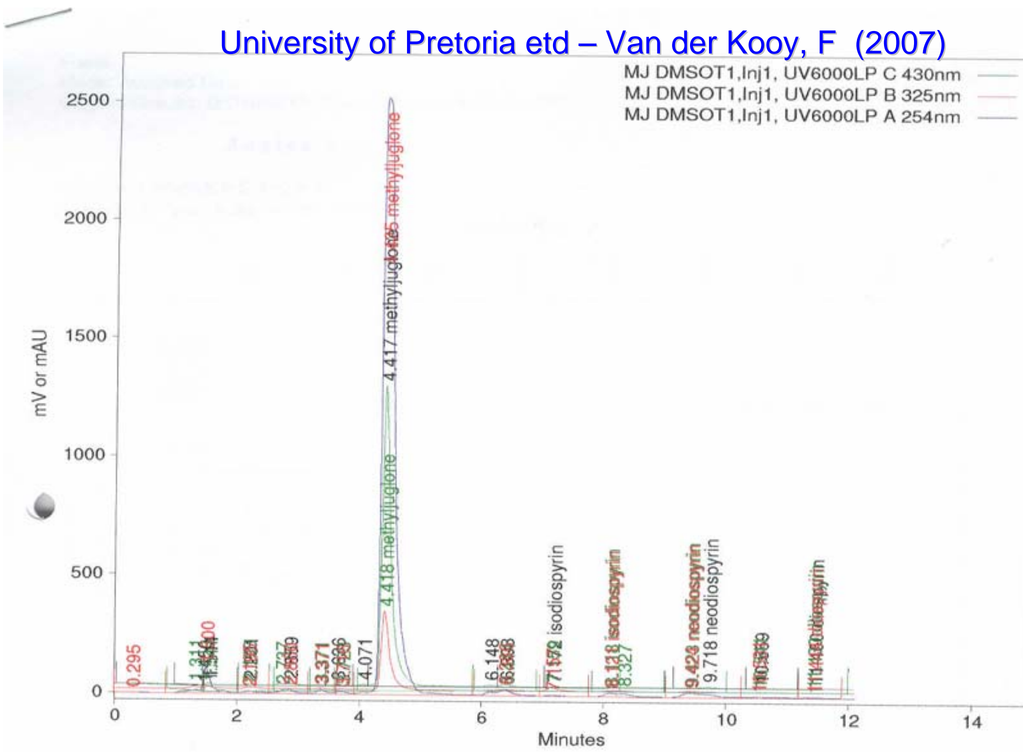


Fig. 5.1: HPLC chromatogram of 7-methyljuglone at a concentration of 10mg/ml in DMSO after 3 minutes.

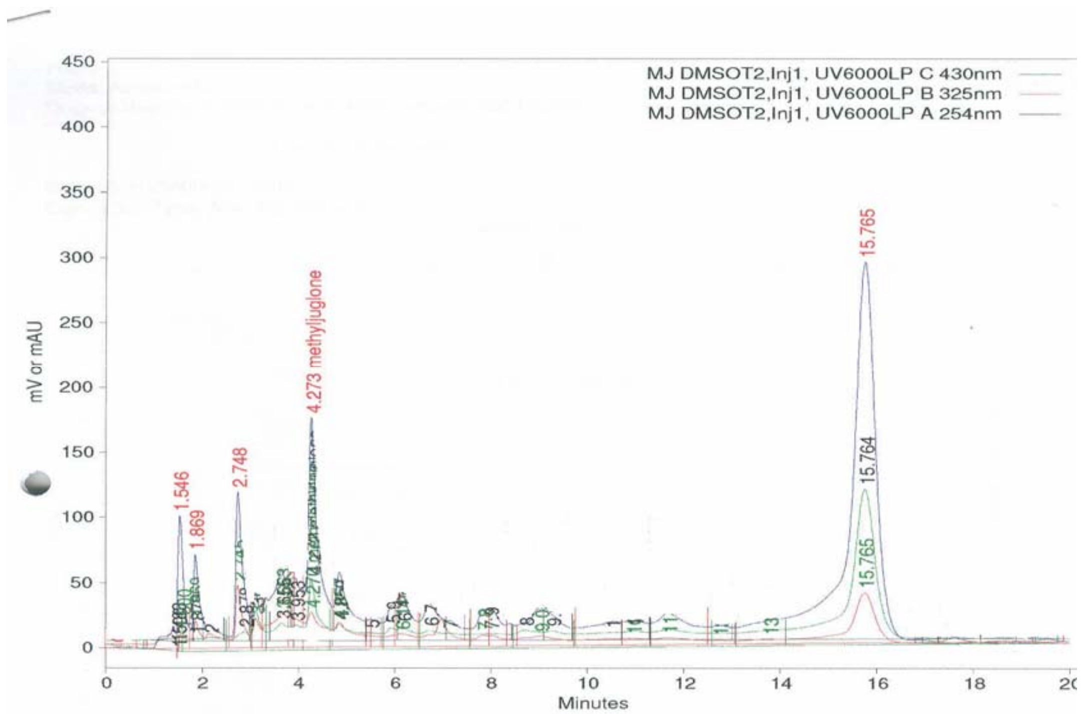


Fig. 5.2: HPLC chromatogram 7-methyljuglone after 24 hours at a concentration of 10mg/ml in DMSO.

University of Pretoria etd – Van der Kooy, F (2007)

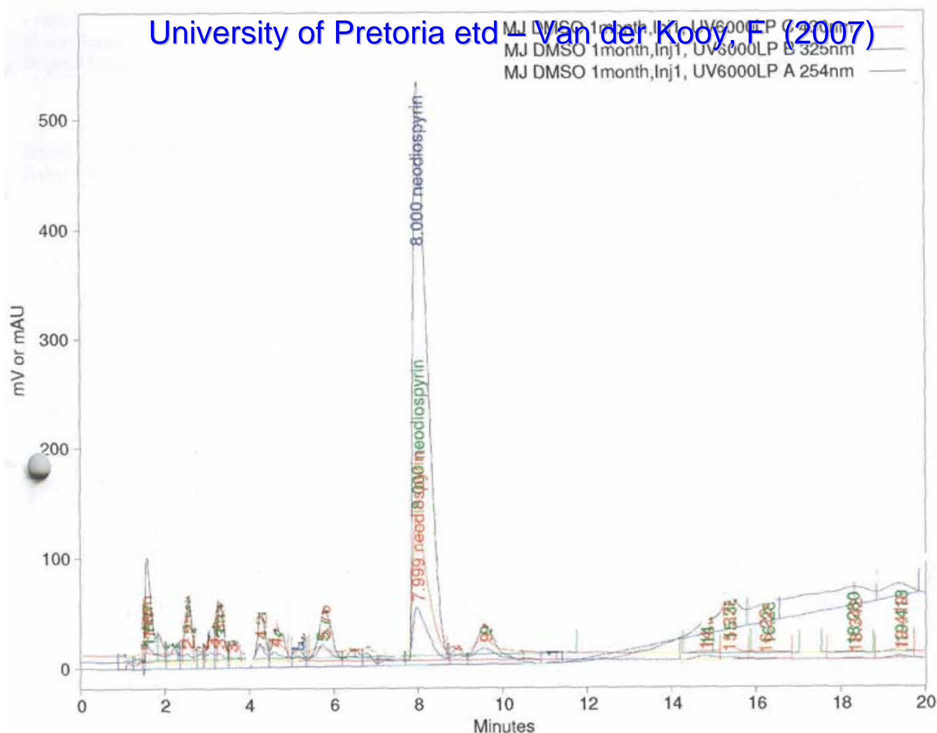


Fig. 5.3: HPLC chromatogram of 7-methyljuglone at a concentration of 1mg/ml in DMSO after 1 month. The breakdown product is different from the 10mg/ml concentration. It appears to be the dimeric compound, neodiospyrin.

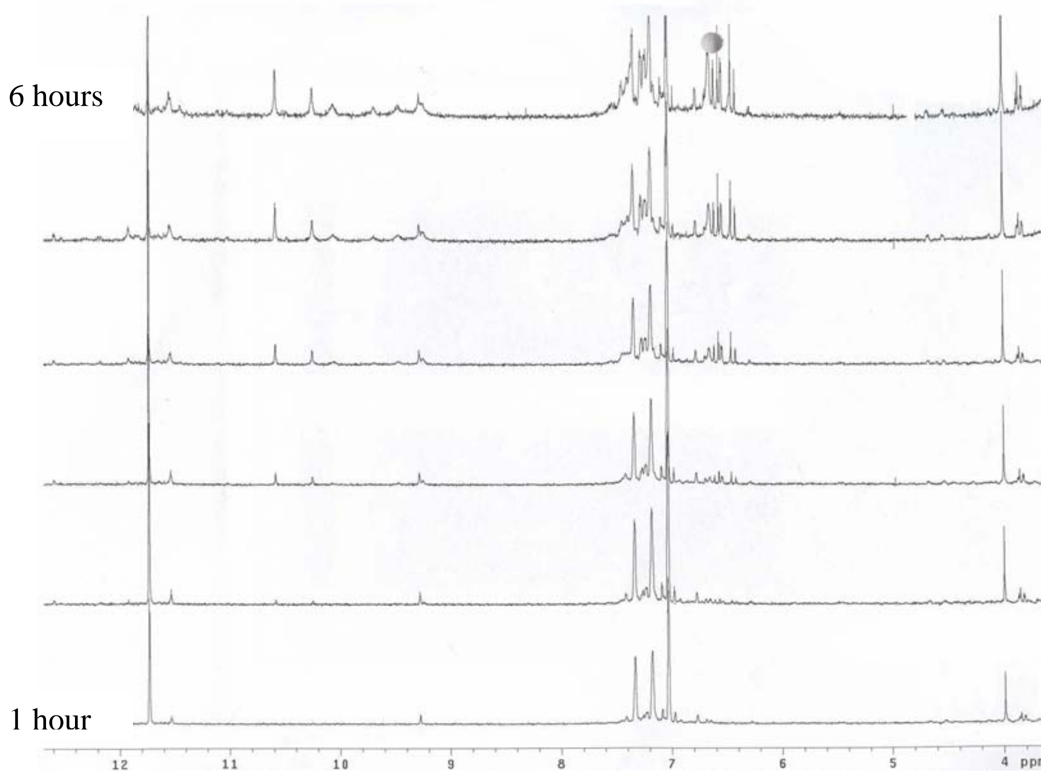


Fig. 5.4: [1] H-NMR kinetic study of 7-methyljuglone in DMSO. The scans were taken 1 hour apart, starting from the bottom.

University of Pretoria etd – Van der Kooy, F (2007)

5.3.2 Stability in BACTEC buffer

The activity of 7-methyljuglone was tested in the BACTEC vial at a concentration of 0.5-5 μg/ml. Due to these low concentrations it was decided to use 100 μg/ml to be able to detect it easily on the HPLC. The compound broke down completely within 80 min. Fig. 5.5-5.7 displays the chromatograms of these tests.

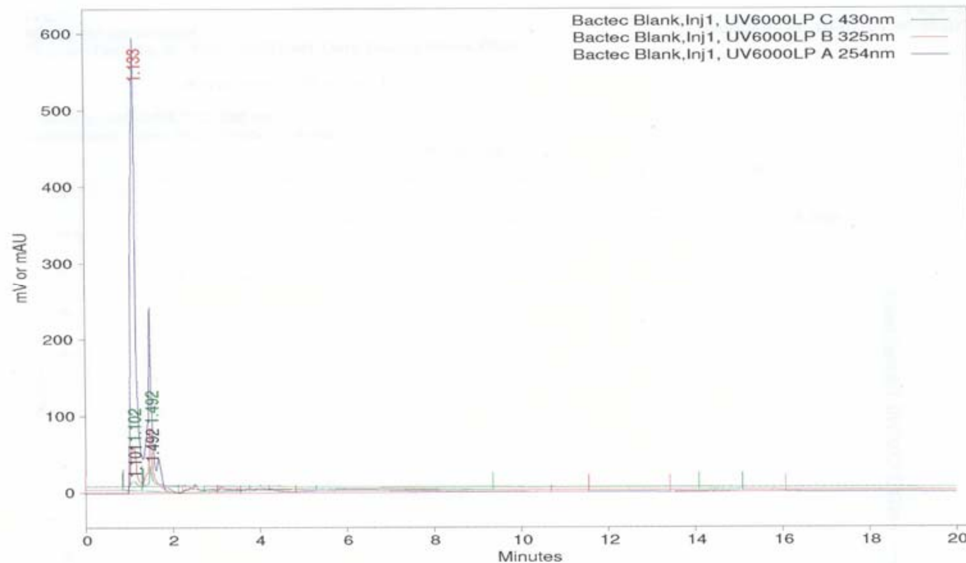


Fig. 5.5:HPLC chromatogram of the BACTEC sample without 7-methyljuglone

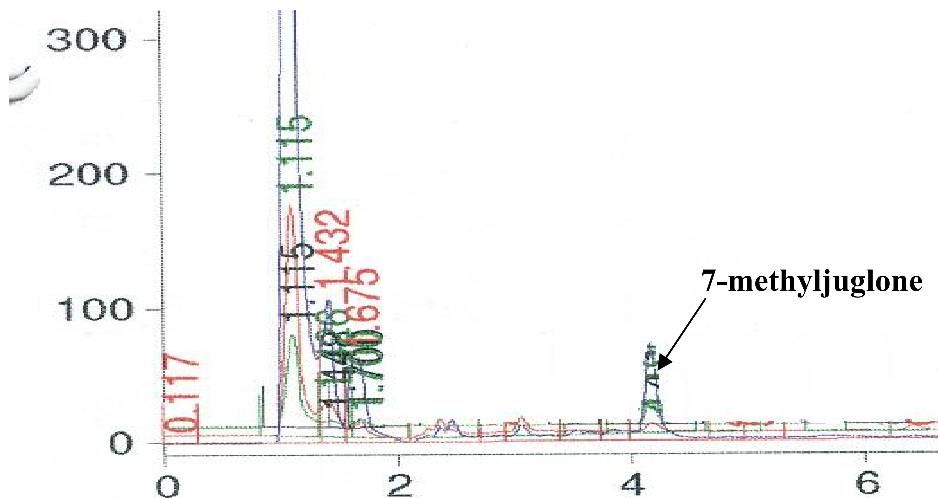


Fig. 5.6: HPLC chromatogram directly after addition of 7-methyljuglone to the BACTEC vial.

Fig 5.7: HPLC chromatogram after 80 minutes. No trace of 7-methyljuglone could be seen. The expanded region as indicated verifies that 7-methyljuglone had broken down.

The concentration of 7-methyljuglone decreased by 40 % during the first 40 min. After 80 min. no traces of the compound could be detected, even after sonication for 15 min before injection.

5.3.3 Stability in vero cell toxicity bioassay buffer

All five NQ's disappeared from the HPLC chromatogram after 2 days. Only menadione and 7-methyljuglone could be found in small amounts after 1 day. After two days all five the compounds broke down completely. Table 5.2 gives the areas of the peaks at different time intervals.

Table 5.2: The time-dependant breakdown of the NQ's in MEM expressed as average percentage area as determined on the HPLC

Compound	Area 0 min	Area 60 min	Area day 1	Area day 2
diospyrin	100	46 ± 2.3	<1	<1
juglone	100	93 ± 1.1	<1	<1
menadione	100	100 ± 2.0	26 ± 1.0	<1
7-methyljuglone	100	74 ± 1.2	23 ± 1.3	<1
plumbagin	100	<1	<1	<1

5.3.4 Stability in 20% DMSO/ broth mixture

The compounds appeared to remain stable, although it was slightly insoluble. Without sonication before each injection, 7-methyljuglone precipitated at a rate of 30 % per day. The precipitation of diospyrin was slightly larger. Before each injection the samples were sonicated and shaken. No breakdown occurred within four days for both compounds.

5.3.5 Stability in 7H9 Middlebrook broth used for mode of action studies

All five the NQ's slowly disappeared from solution (Table 5.3). The compounds appeared to remain stable for 8 hours after which it started to break down. After 24 hours no trace of the compounds could be found. After sonication there was still no trace of the NQ's.

Table 5.3: The time-dependant breakdown of the NQ's in 7H9 Middlebrook broth expressed as average percentage area as determined on the HPLC

Compound	Area 0 min	Area 60 min	Area 8 hours	Area day 1
diospyrin	100	98 ± 1.3	95 ± 0.9	<1
juglone	100	96 ± 2.1	93 ± 0.7	<1
menadione	100	99 ± 0.6	89 ± 1.2	<1
7-methyljuglone	100	100 ± 0.4	92 ± 1.9	<1
plumbagin	100	96 ± 1.2	91 ± 2.1	<1

5.4 Discussion and conclusions

The stability of the NQ's in DMSO gave very perculiar results. It appears that the rate of breakdown is concentration dependant. diospyrin, juglone and 7-methyljuglone were very unstable in this organic solvent. The structural differences between the compounds do not give any clues as to way it is unstable. The substituents on the compounds, a methyl group and hydroxyl group are both *ortho*- and *para* directing activators. A methyl substituent is weakly electron donating through the inductive effect. The hydroxyl substituent is weakly electron withdrawing through the inductive effect, but also strongly electron donating through the resonance effect. Dimethyl sulfoxide is a widely used solvent. It oxidises primary and secondary alcohols to the corresponding ketone or aldehyde groups in the Swern oxidation process. DMSO must however first be activated by an acid chloride. It is therefore unsure why only these three compounds are unstable.

The BACTEC vial, which is used in the determination of the MIC against *M. tuberculosis*, contains ^{14}C-labelled palmitic acid. In addition, the Middlebrook 7H12 agar contains various salts and micro-elements. It also contains catalase and pyridoxine. Catalase is an important detoxifying agent for aerobic bacteria, but unfortunately will also react with NQ's. Pyridoxine has a similar function and this might be the explanation for the disappearance of some of the NQ,s. This will in effect create a two-fold dilemma. The bacteria generate toxic hydrogen peroxide, which the catalase detoxifies. If the NQ's block this enzyme, the bacteria can inhibit further growth and replication by their normal metabolism. The other consideration is that the compounds might be far more active than suspected. If the catalase or pyridoxine inactivate most of the 7-methyljuglone**,** then the few molecules that are able to reach the bacterial cell, might kill it immediately. In effect the MIC should then be considerably lower, unless the breakdown products are also antimycobacterial.

The analysis of the 7H9 Middlebrook broth with 5% glycerol and 20% DMSO appeared to be stable for at least four days. The stock solutions were prepared and administered daily for 4 days. A potential problem of precipitation did occur. If the sample is not shaken thoroughly before administration, the dose might be too low or too high, depending on how deep the syringe was in the mixture. If it was subtracted from the top part a low concentration would have been administered. At the bottom a far higher concentration would have been administered. This might have caused toxic affects in the mice.

The toxicity bioassay medium also showed that the compounds are broken down quite quickly. No proof could be found that the NQ's might react to the ingredients in MEM. It is therefore possible that due to the hydrophobic nature of the compounds it might precipitate. The microtitre plates are not shaken during the bioassay and this might cause the compounds to precipitate onto the cells and in effect have a dramatic increase in concentration.

The media used for the mode of action studies, 7H9 Middlebrook broth with OADC growth supplement, had a similar effect as the BACTEC analysis. The compounds however only disappeared after 24 hours. The mode of action studies (Chapter 8) were therefore only continued for 12-14 hours after the addition of the compounds.

In conclusion it can be said that in three of the solvents some of the NQ's broke down quite rapidly. The 7H9 Middlebrook broth that was used for the mice work showed that the

compounds were stable for the required 4 days. The solution did turn brownish after day 5 and continued to darken with time. Eventually the compounds did also break down in this solvent. These experiments emphasise the importance of stability studies. The MIC of our lead compound in the BACTEC experiments might therefore be a result of inactivation by catalase, but it might also be far more active than suspected. The toxicity bioassay showed that the compounds might be less toxic due to precipitation or that one of the breakdown products is the toxic principle.

5.5. References

Crawford, J. M. & Braunwald, N. S. (1991). Toxicity in vital fluorescence microscopy: effect of dimethylsulfoxide, Rhodamine-123, and DiI-low density lipoprotein on fibroblast growth in vitro. *In Vitro Cellular & Developmental Biology: Animal*. 27(8), 633-8.

Jenkins, P.A. (1998). The microbiology of tuberculosis. In: Clinical tuberculosis, ed. P.D.O. Davies, pp. 72-75. Chapman & Hall Medical, London.

Lipinski, C. A. (2004). Solubility in water and DMSO: Issues and potential solutions. *Biotechnology: Pharmaceutical Aspects 1 (Pharmaceutical Profiling in Drug Discovery for Lead Selection)*. 93-125.

Middlebrook, G., Reggiards, Z. & Tigertt, W.D. (1977). Automable radiometric detection of growth of *Mycobacterium tuberculosis* in selective media. *American Review of Respiratory Diseases*. 115: 1067-1069.

Soerbye, H., Guttu, K., Gislason, H., Grong, K. & Svanes, K. (1993). Gastric mucosal injury and associated changes in mucosal blood flow and gastric fluid secretion caused by dimethyl sulfoxide (DMSO) in rats. *Digestive Diseases and Sciences*. 38(7), 1243-50.

University of Pretoria etd – Van der Kooy, F (2007)

Chapter 6

Toxicity of naphthoquinones

6.1 Introduction **89**

6.2 Materials and methods **89**

6.2.1 Materials **89**

6.2.1.1 Culturing of vero monkey kidney cells **89**

6.2.1.2 Toxicity of 7-methyljuglone and diospyrin in mice **90**

6.2.1.3 Toxicity of 7-methyljuglone in *Musca domestica* **90**

6.2.2 Methods **90**

6.2.2.1 Determination of the IC50 of naphthoquinones on vero cells **90**

6.2.2.2 Toxicity of 7-methyljuglone and diospyrin in mice **91**

6.2.2.3 Toxicity of 7-methyljuglone in *Musca domestica* **91**

6.3 Results **91**

6.3.1 Determination of the IC50 of naphthoquinones on vero cells **91**

6.3.2 Toxicity of 7-methyljuglone and diospyrin in mice **92**

6.3.3 Toxicity of 7-methyljuglone in *Musca domestica* **93**

6.4 Discussion and conclusions **95**

6.5 References **98**

University of Pretoria etd – Van der Kooy, F (2007)

University of Pretoria etd – Van der Kooy, F (2007)

Chapter 6

Toxicity of naphthoquinones

6.1 Introduction

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 (Timbrell, 1996). Various research papers have been published on the biological activity/toxicity of diospyrin and 7-methyljuglone. In the literature review (Chapter 2), various references on the biological activity of naphthoquinones are given. In this chapter the compounds and some derivatives were tested on a vero cell line and also in mice. The toxicity of 7-methyljuglone was in addition also tested on *Musca domestica* to establish a possible mode of action of these compounds in diverse organisms.

6.2 Materials and methods

6.2.1 Materials

6.2.1.1 Culturing of vero monkey kidney cells

Vero cells were cultured in minimal essential medium (Eagle) containing 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10 μg/ml penicillin, 10 μg/ml streptomycin, 0.25 μg/ml fungizone and 10 % fetal bovine serum at 37 °C in a humidified atmosphere with 5 % CO_2. Cells were subcultured in a 1:6 ratio every second to third day after trypsinization of confluent cultures. On day 0, confluent cultures were trypsinized and diluted in complete MEM to a concentration of 1×10^5 cells/ml. In the outer wells of a 96-well plate(s), 200 μl of medium was dispensed. All inner wells received 100 μl (1×10^4 cells) of the cell suspension. The plate(s) was incubated overnight at 37 °C in a humidified atmosphere with 5 % CO_2 (Zheng *et al.,* 2001).

University of Pretoria etd – Van der Kooy, F (2007)

6.2.1.2 Toxicity of 7-methyljuglone and diospyrin in mice

The *in vitro* mice experiments were conducted at the Medical Research Counsel, Pretoria, South Africa. These tests had to be performed by specially trained personnel (Dr. Kobus Venter) and had to be approved by the ethics committee of the MRC. A pilot study was conducted to investigate the toxicity of naphthoquinones *in vivo* in mice. The experiment constituted 140 mice (inbred female BALB/c mice - six to eight weeks of age) divided into six groups.

6.2.1.3 Toxicity of 7-methyljuglone in *Musca domestica*

Approximately 2000 fly larvae (*Musca domestica*) were obtained from the South African Bureau of Standards. Milk powder and sugar, for their feed, were obtained from a local vendor.

6.2.2 Methods

6.2.2.1 Determination of the IC50 of naphthoquinones on vero cells

Stock solutions of twelve pure NQ's **(**8-chloro-7-methyljuglone **(1),** dichlon **(2),** 2,3-dimethoxynaphthoquinone (**3),** diospyrin **(4),** 2-hydroxynaphthoquinone **(5),** isodiospyrin **(6),** juglone **(7),** lapachol **(8),** menadione **(9)** 7-methyljuglone **(10),** neodiospyrin **(11),** plumbagin **(12)** Figure 7.1**)** were prepared in DMSO at 20 mg/ml after which it was immediately diluted 200 times in complete MEM to 100 µg/ml. As a positive control rifampicin was used. To establish the IC50 value for the compounds, the solutions were then serially diluted to obtain eight different concentrations.

A 100 µl solution of the pure compound dilutions were dispensed into cell-containing wells in triplicate of the microtitre plate. The final concentrations of pure compounds in the wells were 0.78, 1.56, 3.13, 6.25, 12.50, 25.00 50.00 and 100.00 µg/ml. Control wells received a final concentration of 0.25 % DMSO in complete medium. Plates were then returned to 37 °C in a humidified atmosphere with 5 % CO_2 for another 3 days. On day 4, 5 µl of XTT reagent was added to the wells and incubation commenced for another 1-4 hrs. The optical densities of the wells were then measured at 450 nm (690 nm reference wavelength) with an Eliza plate reader.

University of Pretoria etd – Van der Kooy, F (2007)

6.2.2.2 Toxicity of 7-methyljuglone and diospyrin in mice

Inbred female mice were treated with the NQ's for five days per week over four weeks. All agents were administered by oral gavage at a dose of 6.25, 12.50 and 25.00 mg/kg of body mass for each of **(4)** and **(10).** Izoniazid was administered at similar concentrations.

6.2.2.3 Toxicity of 7-methyljuglone in *Musca domestica*

Twenty flies were placed in 1 L glass containers, which were covered by mesh cloth. In the containers a bowl of water, sugar and milk powder (mixed with **(10)**) was placed. The concentration of **(10)** mixed with the milk powder were 0.1, 1.0, 10.0 and 20.0 mg/g. Two control groups were used. One group received only the water, sugar and milk powder (without the compound) while the other group received only water (to determine how long the flies would survive without food). The flies were routinely monitored for a period of 2 weeks.

In another experiment 200 flies were placed in a container and the milk powder containing different concentrations of **(10)** was placed at random in the container. It was noted by hourly observation (during the day) which concentration was visited the most and the amount of milk powder that had been consumed after a 2 week period calculated.

6.3 Results

6.3.1 Determination of the IC50 of naphthoquinones on vero cells

(4) and **(10)** did not show cytotoxicity to vero cells at concentrations below their MIC (0.5 mg/ml and 8.0 mg/ml, respectively) *in vitro* (Table 6.1). Most of the compounds showed rather high toxicity when compared to their activity (the activity results are presented in Chapter 7). It does however give valuable information about the active site of the molecule. This will be discussed in the next chapter.

University of Pretoria etd – Van der Kooy, F (2007)

University of Pretoria etd – Van der Kooy, F (2007)

Table 6.1. The cytotoxicity of naphthoquinones on vero cells in nM/ml and µg/ml

Sample	IC_{50}[a] nM/ml	IC_{50}[a] µg/ml
(1)	11.01	2.45 ± 0.10
(2)	128.20	25.00 ± 0.26
(3)	257.73	50.00 ± 2.43
(4)	41.44	17.78 ± 1.69
(5)	287.36	50.00 ± 2.12
(6)	80.21	6.25 ± 1.83
(7)	6.90	3.13 ± 0.46
(8)	413.20	100.00 ± 4.23
(9)	37.97	3.13 ± 1.70
(10)	6.54	1.23 ± 0.36
(11)	86.04	12.5 ± 3.71
(12)	16.49	3.13 ± 0.10
Rifampicin (control)	102.99	84.76 ± 3.45

[a]50% inhibitory concentration

6.3.2 Toxicity of 7-methyljuglone and diospyrin in mice

The preliminary toxicity results showed that **(10)** is toxic to mice at a dose of 25 mg/kg and that **(4)** is not harmful at this concentration. This can be seen in Fig 6.1. The mice treated with **(10)** showed a marked decrease in body mass compared to the control. After treatment stopped the mice recovered most of their body mass and none of these mice died. It can be concluded from the preliminary *in vivo* pilot toxicity study that **(4)** was not cytotoxic at a dose of 25 mg/kg (Figure 6.1) and **(10)** at a dose of 6.25 mg/kg.

University of Pretoria etd – Van der Kooy, F (2007)

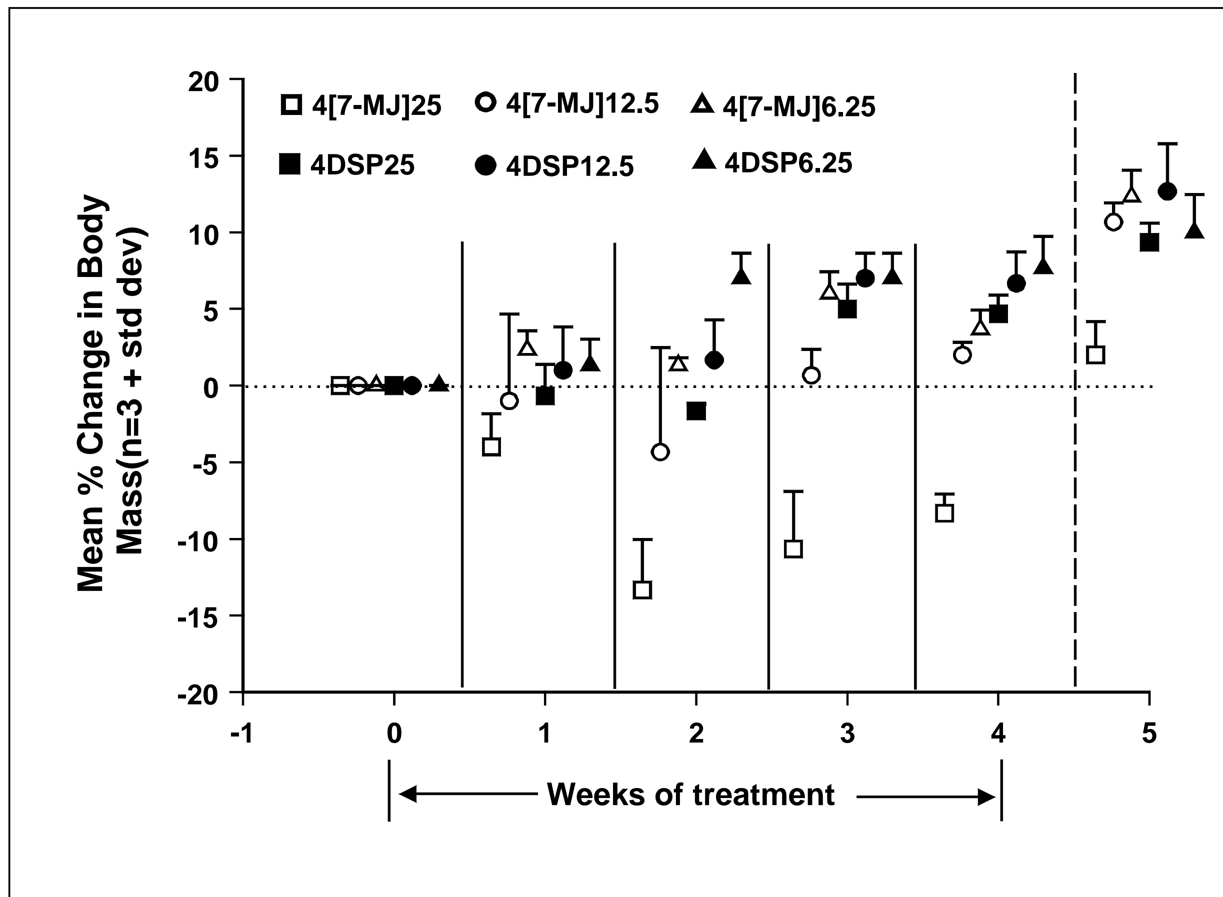


Fig 6.1. Tolerance of (10) (7-MJ) and (4) (DSP) was tested by oral administration (once daily, five times a week for four weeks) at 25.00, 12.50 and 6.25 mg/kg in uninfected mice. Only mice treated with (10) at 25.00 mg/kg displayed significant ($p<0.05$) loss in body mass during weeks 2 to 4. The izoniazid control group did not show any significant changes in body mass (not shown in figure).

6.3.3. Toxicity of 7-methyljuglone in *Musca domestica*

Table 6.2 gives the results after 2 weeks of monitoring. It is clear from the results that the flies consumed the most milk powder in the control group, K1. The milk powder treated with the lowest concentration of **(10)** was consumed the second most. The total amount of milk powder

University of Pretoria etd – Van der Kooy, F (2007)

University of Pretoria etd – Van der Kooy, F (2007)

consumed decreased further with an increase in concentration of **(10).** The amount of flies that died at the different concentrations of **(10)** might indicate a slight toxicity, but this is not conclusive as the variation in the results are very high. The results indicated that the compound might be acting as an antifeedant, as suggested by Tokunaga (2004).

Table 6.2. The amount of milk powder consumed by the flies after a two week period. The number of dead flies is also indicated. K1=milkpowder without (10), K2= no milkpowder.

Sample	[7-methyl juglone] (mg/g)	Amount consumed (mg)	Dead flies (2 weeks)	Average consumed (mg)	Average number of dead flies
1	20.0	38.3	0	77.8 ± 37.1	1.30 ± 1.53
2	20.0	111.8	3		
3	20.0	83.4	1		
4	10.0	102.1	2	87.1 ± 19.7	2.00 ± 1.00
5	10.0	94.4	3		
6	10.0	64.7	1		
7	1.0	60.0	0	114.8 ± 75.0	0.33 ± 0.56
8	1.0	84.0	0		
9	1.0	200.3	1		
10	0.1	206.7	0	155.7 ± 50.7	0.00
11	0.1	105.4	0		
12	0.1	155.1	0		
K1	-------	252.4	0	252.4	0.00
K2	-------	--------	all	0.0	15.00

The results of the experiment that contained milk powder mixed with **(10)** which was placed at random in one container, are given in Table 6.3. The results clearly indicate that the flies prefer the milk powder with no addition of **(10).** The lowest concentrations are also visited more than the higher concentrations. This might indicate that **(10)** acts as an antifeedant.

University of Pretoria etd – Van der Kooy, F (2007)

University of Pretoria etd – Van der Kooy, F (2007)

Table 6.3: Amount of fly visits (observed at random) and the amount of milk powder consumed at different concentrations of (10) mixed with milk powder.

[(10)] in milk powder in mg/g	Milk powder consumed in mg	Total amount of flies that visited
0.0	300.5 ± 22.5 (96%)	22.5 ± 4.5
0.1	238.7 ± 53.5 (80%)	26.0 ± 20.0
1.0	193.4 ± 20.0 (63%)	22.0 ± 9.0
10.0	129.1 ± 17.0 (43%)	1.5 ± 0.5
20.0	123.5 ± 48.5 (41%)	3.0 ± 0.0

6.4. Discussion and conclusions

The toxicity against vero cells gave a clear indication that these naphthoquinones are toxic to cells. The solubility and stability of these compounds in MEM must however be taken into consideration. These compounds have a log P value of approximately 3, which means that they are hydrophobic (**(4)** log P = 4.5, **(10)** Log P = 2.2). This might indicate that the compounds might have precipitated and was absorbed by the cells at a higher dose than was actually meant.

Khambay *et al*. (2003) reported on the toxicity of structurally similar naphthoquinones to *M. domestica*. The MIC for two derivatives of dunnione ranged from 3-4 µg/ml against *M. domestica*. Fig. 6.1 illustrates the chemical structures of the three compounds. The mechanism of action proposed by him involves the inhibition of complex 1 in the electron transport chain (Khambay *et al*., 2003). Our fly experiments indicated that **(10)** is not toxic to *Musca domestica*. It does however influence the amount of milk powder consumed. This probably means that the flies are repelled by the compound. There are reports that the structurally similar compound plumbagin, shows high activity against flies. Most flies died after a topical addition of 10 µg of plumbagin on the flies (Saxena *et al.,* 1996). This compound occurs in the well known Venus fly trap (*Dionaea muscipula*) and its role there has not yet been defined.

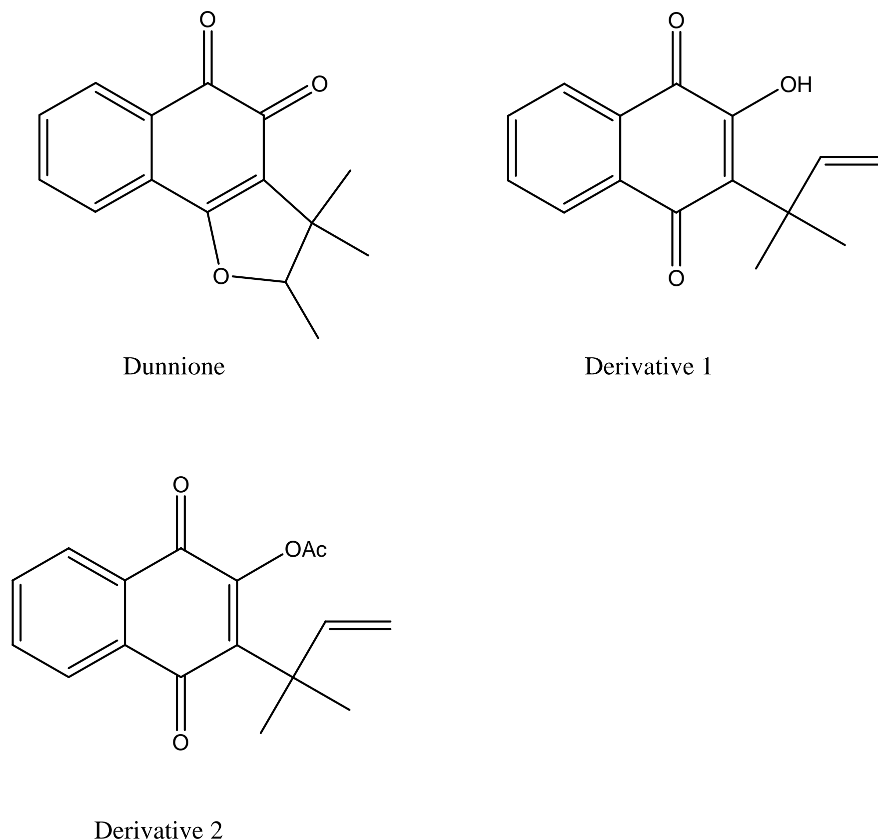


Fig. 6.1: Structures of naphthoquinones which show activity against *M. domestica*

After 2 weeks the flies exposed to the highest concentration did not die at a higher rate than those treated with lower concentrations, or the control group. This could clearly be seen when the flies were given a choice between the different concentrations. It was clearly visible that the most flies visited the control milk powder and the lowest concentration of **(10)** mixed with milk powder.

The toxicity results of all three experiments indicated that the naphthoquinones are toxic to some extent. The conclusions that can be drawn from the vero cells toxicity experiments, is that the cells were probably exposed to a higher concentration than that was meant, due to solubility problems. To overcome this problem will be a rather difficult exercise. Various lipophilic carriers must be tested on cell lines to ensure that it is not toxic to cells at a rather high dosage. The fly experiments indicated that it is not as such toxic to *M. domestica,* but might act as a repellent.

University of Pretoria etd – Van der Kooy, F (2007)

University of Pretoria etd – Van der Kooy, F (2007)

This is rather counter-intuitive as *D. capensis* plants might produce high amounts of the compound to attract insects by means of the bright colour (orange-red) of the leaves. Does the plant use this compound to kill only specific types of insects (that reacts to the colour attraction) and to repel others that might damage the leaf lamina? To solve this question experiments have to be set up that test various different kinds of insects. This will give answers to the use of this compound to the plant. The toxicity of **(4)** and **(10)** in mice showed that the compounds were toxic at the doses administered. The insolubility of the compounds in the carrier is however a cause for concern. The compounds did start to precipitate out of solution after a couple of hours.

University of Pretoria etd – Van der Kooy, F (2007)

6.5. References

Ibrahim, A., Boutros, A. & McDougall, J. B. (1955). Chemotherapy in a Cairo Chest Clinic; a preliminary report on the methods adopted in assessing the value of izoniazid , streptomycin and paraminosalicylic acid in 122 cases of pulmonary tuberculosis. *The British journal of tuberculosis and diseases of the chest.* 49(1), 38-49.

Khambay, B. P. S., Batty, D. Jewess, P. J., Bateman, G. L. & Hollomon, D. W. (2003). Mode of action and pesticidal activity of the natural product dunnione and of some analogues. *Pest Management Science.* 59(2), 174-182.

Saxena, B. P., Tikku, K. & Thappa, R. K. (1996). Plumbagin , a sterilant for *Musca domestica* males. A light and electron microscopic study of its effect on testis. *Proceedings of the Indian National Science Academy, Part B: Biological Sciences*. 62(3), 199-205.

Timbrell, J.A. 1991. Principles of biochemical toxicology. 2nd ed. pp 7-9. Taylor & Francis, London.

Tokunaga, T., Takada, N & Ueda, M. (2004). Mechanism of antifeedant activity of plumbagin, a compound concerning the chemical defence in carnivorous plants. *Tetrahedron letters*. 45(38), 7115-7119.

Zheng, Y. T., Chan, W. L., Chan, P., Huang, H. & Tam, S. C. (2001). Enhancement of the anti-herpetic effect of trichosanthin by acyclovir and interferon. FEBS Letters. 496, 139-142.

University of Pretoria etd – Van der Kooy, F (2007)

Chapter 7

Structure-activity relationship of naphthoquinones

7.1 Introduction 100

7.2 Materials and methods 101

7.2.1 MIC determination 101

7.2.2 Toxicity bioassay 103

7.3 Results 103

7.3.1 MIC and toxicity determination 103

7.3.2 Structure-activity relationship 104

7.4 Discussion and conclusions 105

7.5 References 106

Chapter 7
Structure-activity relationship of naphthoquinones

7.1 Introduction

The aim of any structure-activity relationship study should be to find the most potent and least toxic compound. To be able to find such a compound a structure-activity relationship (SAR) study must be carried out. 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 (Silverman, 2004).

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 SAR studies is therefore to synthesise as many analogs as possible from the lead compound and testing the effect the structure has on the potency as well as the toxicity.

Several structurally related compounds (isolated, synthesised or commercially obtained) have been tested for potency against TB to determine the active site (pharmacopore) of the lead compound. In total thirteen compounds have been tested for activity (with the use of the radiometric BACTEC bioassay) and for toxicity with the use of vero cells.

7.2 Materials and methods

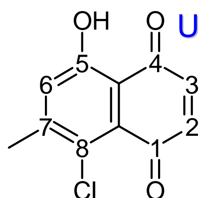
7.2.1 MIC determination

The naphthoquinones used during these experiments included the isolated compounds: diospyrin **(4),** isodiospyrin **(6),** neodiospyrin **(11),** shinanolone **(13),** mamegakinone (isolated from *E. natalensis*) and plumbagin **(12)** (isolated from *Plumbago auriculata*). The synthetic compounds were 8-chloro-7-methyljuglone **(1)** and 7-methyljuglone **(10).** The compounds obtained from Sigma-Aldrich includes: dichlon **(2),** 2,3-dimethoxynaphthoquinone **(3),** 2-hydroxynaphthoquinone **(5),** juglone **(7),** lapachol **(8)** and menadione **(9)** (Fig. 7.1).

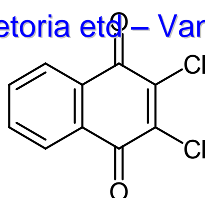
A susceptible strain of *Mycobacterium tuberculosis* H37Rv, obtained from the American Type Culture Collection (Rockville, MD, USA), was used to investigate the activity of the naphthoquinones. The 7H12 Middlebrook TB medium (Middlebrook *et al.,* 1977) used during these studies, consisted of an enriched Middlebrook 7H9 broth base supplemented with bovine serum albumin, catalase, casein hydrolysate and ^{14}C-labelled substrate (palmitic acid) as a source of carbon. Growth of the mycobacterium leads to the consumption of the carbon source, with subsequent release of labelled $^{14}CO_2$. This labelled CO_2 moves into the atmosphere above the medium in the sealed vial and the BACTEC TB 460 instrument detects the amount of $^{14}CO_2$ and records it as a growth index (GI) on a scale of 0-999 (Heifets and Good, 1994).

For the MIC determination, a vial containing 7H12 Middlebrook TB medium was inoculated with homogenised cultures in a special diluting fluid (Middlebrook-Dubos 7H9 broth having the no.1 McFarland standard optical density). When growth in this vial reached a GI reading of 400-500, the 7H12 broth culture was used undiluted to inoculate a set of vials. The isolated compounds, dissolved in dimethylsulfoxide (DMSO), were added into the vials to give a final concentration of 1 % DMSO. The positive drug controls ethambutol, izoniazid and rifampicin and a 1 % DMSO control were used.

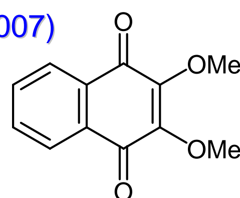
University of Pretoria etd – Van der Kooy, F (2007)



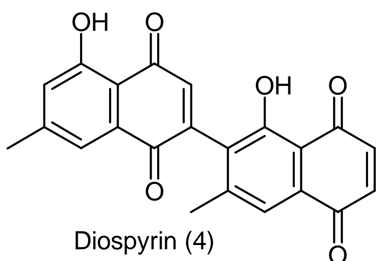
Chloro-7-methyljuglone (1)



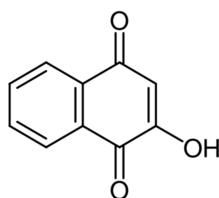
Dichlon (2)



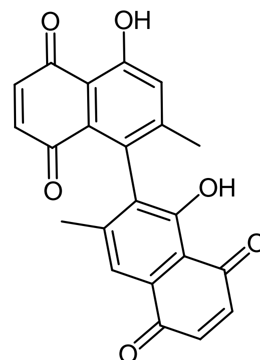
2,3-dimethoxynaphthoquinone (3)



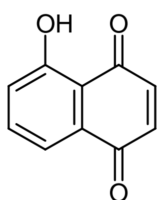
Diospyrin (4)



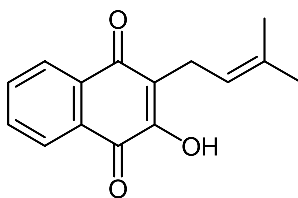
2-hydroxynaphthoquinone (5)



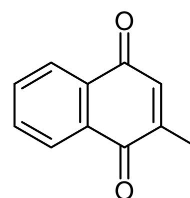
Isodiospyrin (6)



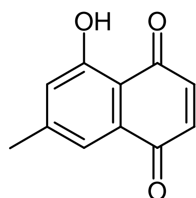
Juglone (7)



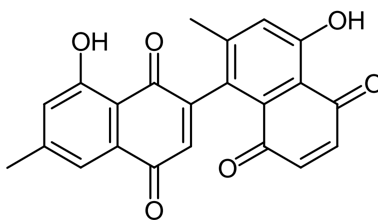
Lapachol (8)



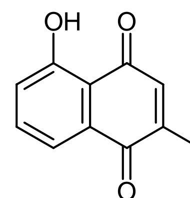
Menadione (9)



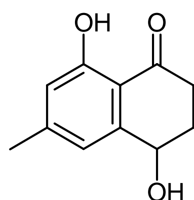
7-methyljuglone (10)



Neodiospyrin (11)



Plumbagin (12)



Shinanolone (13)

Fig. 7.1: The chemical structures of the compounds used for SAR studies. The numbering system is indicated for chloro-7-methyljuglone.

Inoculated vials were incubated at 38 ºC and each vial was assayed daily at the same hour until cumulative results were interpretable. The GI value of the control vial was compared with the readings from the vials containing the compounds. The control vial contains a 1:100 dilution of the inoculum and when it reached a reading of 30 the readings were stopped. The difference in GI readings for the last two days (ΔGI) was used. If ΔGI readings of any of the compounds were less than the control vial, that compound was considered to be active. All compounds were tested in triplicate at concentrations of 0.5, 1.0, 5.0, 10.0, 25.0, 50.0, 100.0μg/ml.

7.2.2 Toxicity bioassay

The toxicity bioassay on vero cells was done as described in Chapter 5.

7.3 Results

7.3.1 MIC and toxicity determination

The results of the anti-TB and toxicity of the naphthoquinones are given in Table 7.1 and are expressed in nM/ml. The MIC's of the positive controls: isoniazid, rifampicin and ethambutol were 0.45, 0.151 and 61.27 nM/ml respectively.

Table 7.1: The MIC and toxicity of naphthoquinones used during this study in nM/ml

Compound	MIC	IC50	Selectivity index
(1)	17.90	11.01	0.62
(2)	440.23	128.20	0.29
(3)	458.30	257.73	0.56
(4)	21.40	41.44	1.93
(5)	574.18	287.36	0.50
(6)	26.73	80.21	3.00
(7)	5.75	6.90	1.20
(8)	41.30	413.20	10.00
(9)	29.07	37.97	1.30
(10)	2.66	6.54	2.46
(11)	26.74	86.04	3.20
(12)	5.32	16.49	0.59

(13)	335.00	173.30	0.52

7.3.2 Structure - activity relationship

Fig. 7.1 illustrates the chemical structures of the naphthoquinones. The structure of the naphthoquinones shows that the double ketone motif on carbon 1 and 4 is needed for potency. The activity declines dramatically when the ketone is reduced to the corresponding hydroxyl group (e.g. MIC of **(13)** = 335 nM/ml). The aromaticity of the naphthalene ring also influences the potency, with the reduction of the aromatic ring the activity declines sharply (shinanolone). The presence of the hydroxyl group confirs activity. By blocking carbon 2 and 3 the possibility that a Michaels adduct can form is reduced. Fig. 7.2 indicates the mechanism of a Michaels adduct.

The conjugate nucleophilic addition to α-β-unsaturated carbonyl groups play an important role, and it might be due to interaction via this mechanism with thiol groups (nucleophilic groups) on proteins, that cause the toxicity of these compounds (McMurray, 1996; Lipinsky, personal communication). All the compounds with functional groups on both carbons (C2 and C3) have IC50 values of 128.20 nM/ml and higher. The only compound that showed good activity with these two carbons blocked is lapachol. The other two compounds (**(2)** and **(3)**) lost their activity as well as their toxicity.

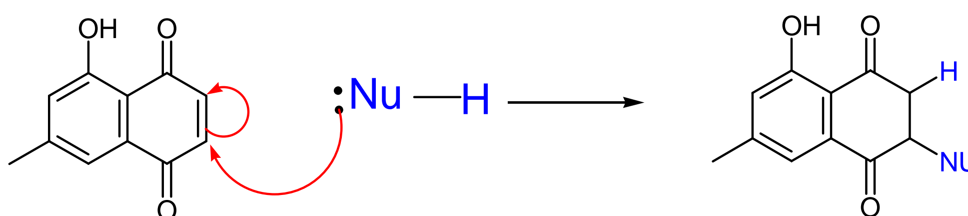


Fig. 7.2. The mechanism of a Michael's reaction.

7.4 Discusion and conclusions

In general the toxicity increases when the activity increases, which would indicate that the pharmacophore and the toxic site are similar. The active site may however be the 1,4 ketone motif with the toxic site the unsaturated unblocked carbon 2 and 3. Due to the above results we are currently in the process of synthesising more compounds with positions C2 and C3 blocked. This will decrease the probability that a Michaels adduct can form while the redox potential of the ketone groups remain the same.

Lapachol is one of the compounds that showed the most promise. The compound will be derivatised further with the addition of hydroxyl functional groups at different positions which might increase its activity further. The instability of the compounds in the toxicity tests and the potency bioassays are a cause for concern. This might yield inaccurate results. The high hydrophobicity of the NQ's increases the problem even further. In the next chapter the possible mode of action is discussed with the redox potential of the ketone groups as the main active mechanism.

The main aim of the SAR studies in future will be to find a compound that will only interact with menaquinone (occurring in mycobacterium) and not with ubiquinone (occurring in mammals). Therefore the structure would have to be modified further in order to enhance the specific activity and to decrease the toxicity.

7.5 References

Heifets, L.B. & Good, R.C. (1994). Current laboratory methods for the diagnoses of tuberculosis. In: Tuberculosis: pathogenesis, protection and control, ed. B.R. Bloom, pp 85-108. ASM Press, Washington, DC.

McMurry, J. (1996). Organic chemistry. 4th ed. pp 914-917, Brookes/Cole Publishing, USA.

Middlebrook, G., Reggiards, Z. & Tigertt, W.D. (1977). Automable radiometric detection of growth of *Mycobacterium tuberculosis* in selective media. *American Review of Respiratory Diseases*. 115: 1067-1069.

Silverman, R.B. (2004). The organic chemistry of drug design and drug action. pp 21-22, Elsevier Academic Press, USA.

University of Pretoria etd – Van der Kooy, F (2007)

University of Pretoria etd – Van der Kooy, F (2007)

Chapter 8

The mode of action of naphthoquinones in *Mycobacterium smegmatis*

8.1 Introduction 108

8.2 Materials and methods 113

8.2.1 Activity against *M. smegmatis* 113

8.2.2 *M. smegmatis* cultures 113

8.2.3 Extraction of M. smegmatis cells 114

8.2.4 HPLC analysis 114

8.3 Results 114

8.3.1 Activity against *M. smegmatis* 114

8.3.2 *M. smegmatis* cultures 116

8.3.3 HPLC analysis 116

8.4 Discussion and conclusions 118

8.5 References 121

University of Pretoria etd – Van der Kooy, F (2007)

University of Pretoria etd – Van der Kooy, F (2007)

Chapter 8

The mode of action of naphthoquinones in *Mycobacterium smegmatis*

8.1 Introduction

The focus of this chapter was on the mechanism of action of our lead compound, 7-methyljuglone in *Mycobacterium smegmatis*. The non-pathogenic *M. smegmatis* is usually used as an indicator species in laboratories that are not equipped to culture the pathogenic *M. tuberculosis*. Another species that are sometimes used as a substitute is *M. aureum*. The possible implication of using indicator species, is that the lead compound might not show any activity, as is the case with the known TB drug Izoniazid. Izoniazid shows very good activity against *M. tuberculosis,* but is not active against *M. smegmatis*. The compounds should therefore first be tested for activity against *M. smegmatis,* before continuing with the mode of action experiments.

No literature could be found on the antimycobacterial mode of action of 7-methyljuglone**.** There are however various reports on the biological activity in other organisms e.g. termicidal activity (Carter *et al.,* 1978) and antifungal activity (Steffen & Peschel, 1975). It appears from the literature that the biological activity of this compound is very broad, which will probably classify it as a non-specific binding site "drug". Published reports on the other NQ's showed that diospyrin and isodiospyrin bind or inhibit topoisomerase I and therefore might be useful as anticancer drugs. It is also speculated by Cushion *et al*. (2000) that diospyrin can bind to the mithochondrial electron transport chain. The literature review (Chapter 1) gives a complete list of activities associated with the naphthoquinones while Table 8.1 recaps the most important mode of action publications that could be found for the naphthoquinones. It can be concluded from the literature reviews that the mode of action of the lead compound remains unknown. Structurally similar naphthoquinones do however have speculative mode of actions including respiration inhibition (juglone).

University of Pretoria etd – Van der Kooy, F (2007)

Table 8.1: Mechanism of action associated with naphthoquinones as found in literature

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

According to Weinstein et al. (2005), M. tuberculosis is an obligate aerobe but can survive for long periods under low oxygen conditions. Fig. 8.1 illustrates an electron transport chain system. The mycobacterial electron transport chain occurs in the cell wall of M. tuberculosis and consists of 4 complexes. In complex 1, NADH dehydrogenase, receives electrons from FAD and NADH. The redox potential of this complex is lower than that of complex 2. The electrons are transported to the second complex by the mobile menaquinone. Menaquinone becomes reduced to menaquinol and shuttles the electrons to complex 3, the cytochrome bc_1 complex (under anaerobic conditions the electrons are shuttled to the cytochrome bd complex). During the transfer of electrons the menaquinol becomes oxidised back into menaquinone. From the cytochrome bc_1 complex the electrons flow to cytochrome aa_3 and convert O_2 into water. The resulting H^+ gradient phosphorolates ADP to form ATP in the ATP synthase complex (Matsoso et al. 2005).

University of Pretoria etd – Van der Kooy, F (2007)

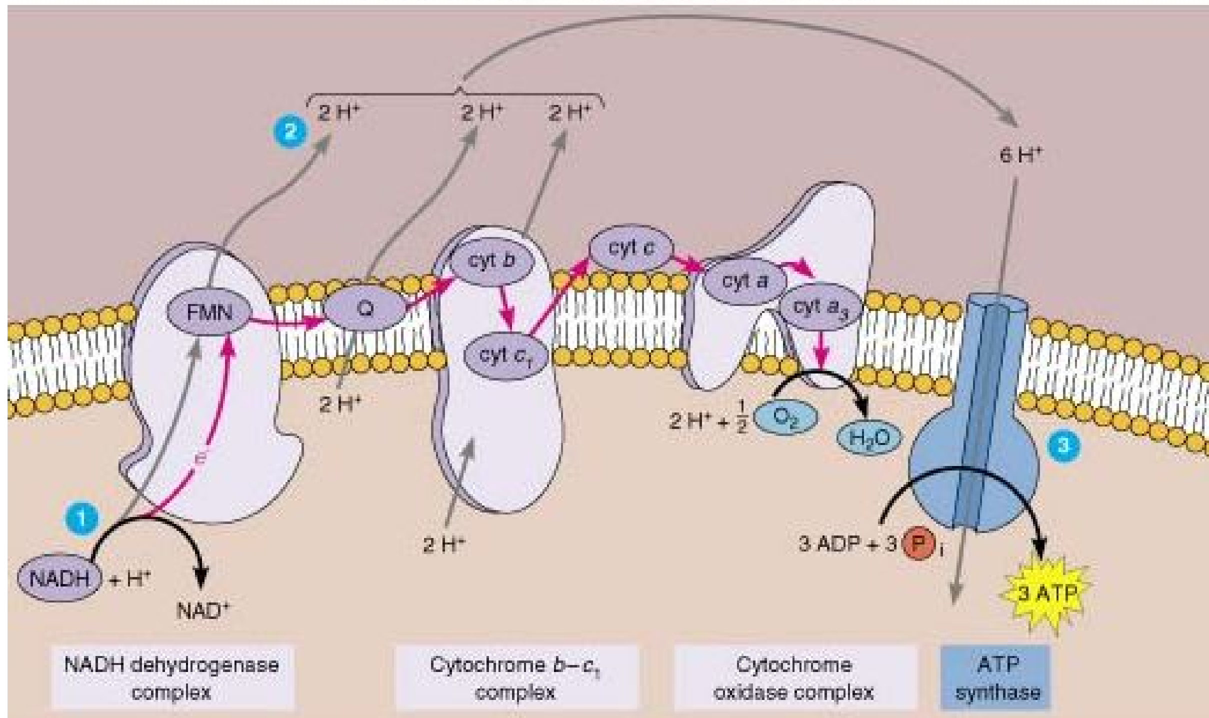


Fig 8.1: The electron transport chain with its different complexes. Q represents ubiquinone. Mycobacterium tuberculosis has menaquinone in this position (http://www.columbia.edu/cu/biology/courses/c2005/handouts/etccomplexes.jpg).

The similarities between 7-methyljuglone and menaquinone shows a possible interference of the electron transport system in mycobacteria (Fig. 8.2). Menaquinone consists of a 1,4-napthoquinone skeleton with two side chains attached on carbon 2 and 3. The lengh of the isoprene unit on carbon two differs between species but consists of 9 units in *M. tuberculosis* (45 carbons). The role of the side chain is to make the compound hydrophobic enough so that it can move inside the cell wall. Ubiquinone (found in humans) consists of a phenolic skeleton with similar side chains on carbon 2 and 3. This might indicate that menaquinone is an attractive drug target because it lacks a human equivalent.

The mode of action might occur in one of three ways. 7-Methyljuglone can enzymatically be incorporated into a menaquinone-like compound with the subsequent interferences in the chain due to an altered redox potential. This can be achieved by competitive binding of 7-methyljuglone to the enzymes producing menaquinone. Fig 8.3 illustrates the formation of menaquinone and where 7-methyljuglone might interfere (indicated with arrows).

University of Pretoria etd – Van der Kooy, F (2007)

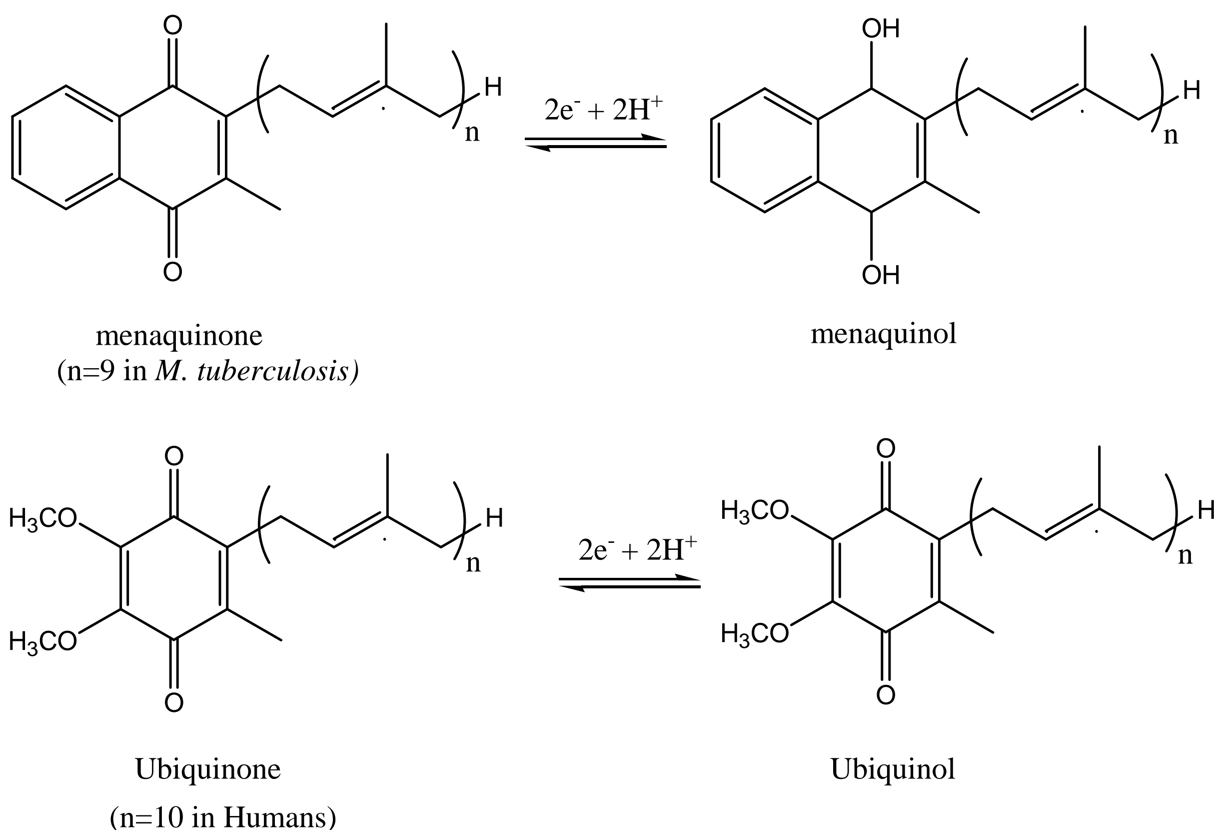


Fig. 8.2: The structural difference between the redox reactions of the mycobacterial menaquinone and ubiquinone found in humans (Truglio *et al*., 2003)

7-Methyljuglone might compete with *men* A or the *men* G enzyme and thereby either stops the production of menaquinone or it might be enzymatically incorporated into a menaquinone-like compound with the addition of a long carbon sidechain. It might therefore also be possible to stop the addition of the methyl group on carbon three. The result of this interference would be two-fold. Firstly menaquinone production can be stopped or the tempo of production might be influenced. Secondly, 7-methyljuglone can be incorporated into the role of menaquinone. This will have an influence on the rate of electron transport due to a different redox potential that this new compound will have. This will effectively either increase respiration or decrease respiration.

University of Pretoria etd – Van der Kooy, F (2007)

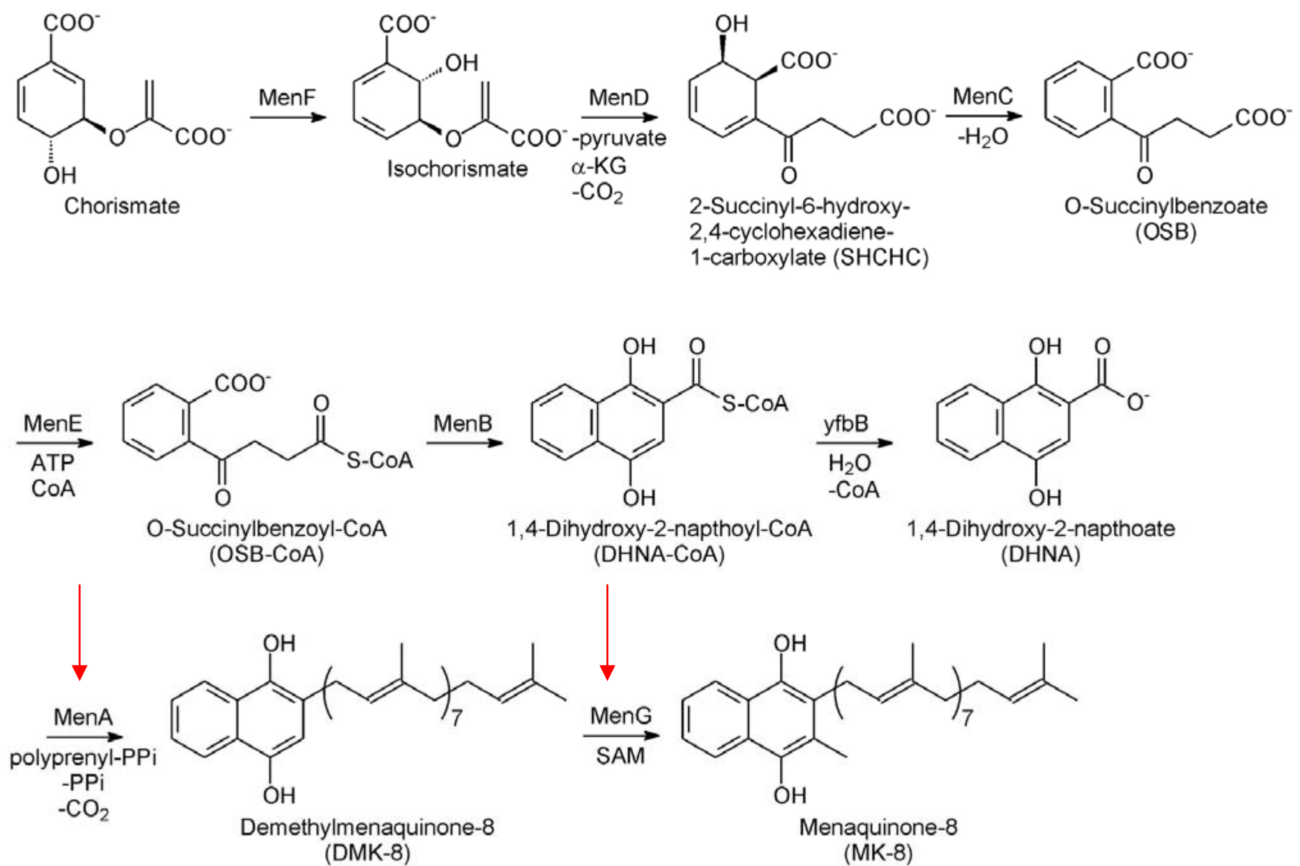


Fig. 8.3 Menaquinone production with the two possible sites of interference indicated with arrows (Truglio *et al*., 2003)

7-Methyljuglone might competitively bind to the menaquinone enzyme, thereby inhibiting menaquinone from forming. This will effectively stop the electron transport chain. 7-Methyljulone might also bind to one of the complexes in the chain and thereby prohibit electrons from flowing down the chain. Either by scavenging electrons from complex 1 or binding to the cytochrome complexes.

During this chapter the influence of some of the NQ's on the mycobacterial electron transport chain was studied in *M. smegmatis.* The mycobacterium was cultured and treated with naphthoquinones. The treated cells as well as a control group was quantitatively extracted and subjected to HPLC analysis.

University of Pretoria etd – Van der Kooy, F (2007)

University of Pretoria etd – Van der Kooy, F (2007)

8.2 Materials and methods

8.2.1 Activity against *M. smegmatis*

The fluorinated 7-methyljuglone, juglone, 7-methyljuglone and plumbagin and the known TB drug izoniazid were chromatographed on normal phase silica gel TLC plates using hexane:ethyl acetate (5:2) as mobile phase. In addition a phase three clinical trial anti-TB drug, moxifloxacin, was also used as a positive control. The volumes of the compounds spotted were 50, 20 and 5 µl of 1 mg/ml solutions. The plates were dried and sprayed with freshly subcultered *M. smegmatis* in 7H9 Middlebrook broth with OADC growth supplement (Begue & Kline, 1972; Eloff, 1998). The plates were incubated at 37 °C for 24 hours after which they were sprayed with INT (2 mg/ml). The plates were incubated for an additional 4 hours before being inspected for activity. The zones in which INT did not give a purplish colour were indicative of activity.

8.2.2 Mechanism of action bioassay

The mycobacterial cultures were obtained from the Medical Research Council in Pretoria. The cultures were sub-cultured in 7H9 Broth (4.5 g / 450 ml) with 2 ml glycerol. The broth was autoclaved and ADC growth supplement added when the broth cooled down to 45 °C. The mycobacteria were placed in a shaker in an incubator at 37 °C in the dark (Weinstein *et al.,* 2005). After 24 hours the mycobacteria were centrifuged at 4000 rpm and the supernatant discarded. Fresh broth was added to the cultures. The sub-culturing was repeated daily for four days in order to obtain a large amount of cells. On day four the medium was removed by centrifugation and the subcultures with fresh medium subdivided into five equal groups. They consisted of a control group and four NQ treated groups. Fresh broth was added while the four NQ's were dissolved in DMSO. The compounds were added to the cultured mycobacteria to achieve a final concentration of 100 µg/ml. The DMSO had a final concentration of 1 %. The control group also contained DMSO.

8.2.3 Extraction of *M. smegmatis* cells

The five cultures were placed on a shaker inside an incubator and after 12 hours they were centrifuged and the amount of cells present in each group weighed, after which it was dried at 100 °C and again weighed (Lester and Crane, 1959). The dried cells were crushed with a mortar and pestle after which they were extracted with dichloromethane and hexane three times each. The extracts were dried on a rotary evaporator and subjected to HPLC analysis.

8.2.4 HPLC analysis

The HPLC system consisted of a Phenomenex RP18 (150 X 4.6mm) column with 100 % acetonitrile as mobile phase (Kroppenstedt, 1982). The column temperature was set to 40 °C with a flow rate of 2 ml/min. The retention time of menaquinone was 25 min. A blank broth sample was injected to see if the broth would cause peak overlap.

8.3 Results

8.3.1 Activity against *M. smegmatis*

The zones of inhibition could clearly be seen on the TLC plates (Fig. 8.4-8.6). INH did not inhibit the growth as was expected.

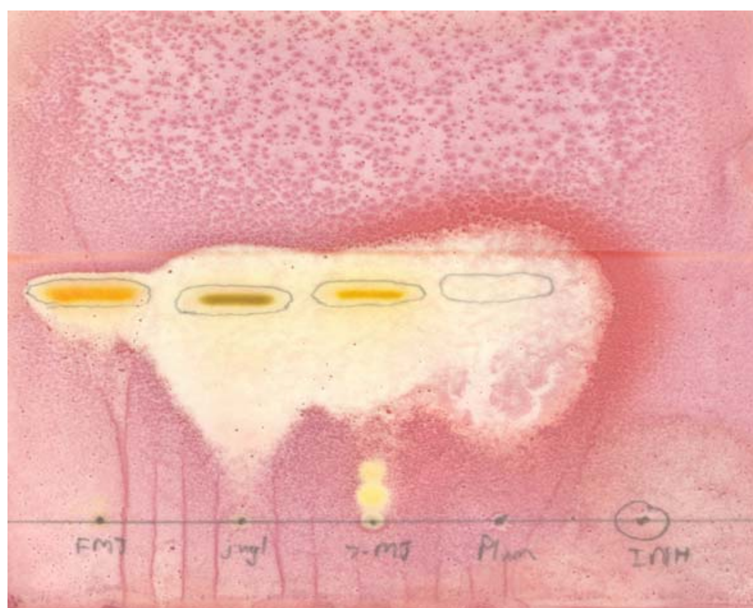


Fig. 8.4: TLC plate with NQ's after spraying with *M. smegmatis*. Lanes 1-5 contain the fluorinated 7-methyljuglone, juglone, 7-methyljuglone, plumbagin and INH at amounts of 50 μg. The compounds are circled in pencil.

University of Pretoria etd – Van der Kooy, F (2007)

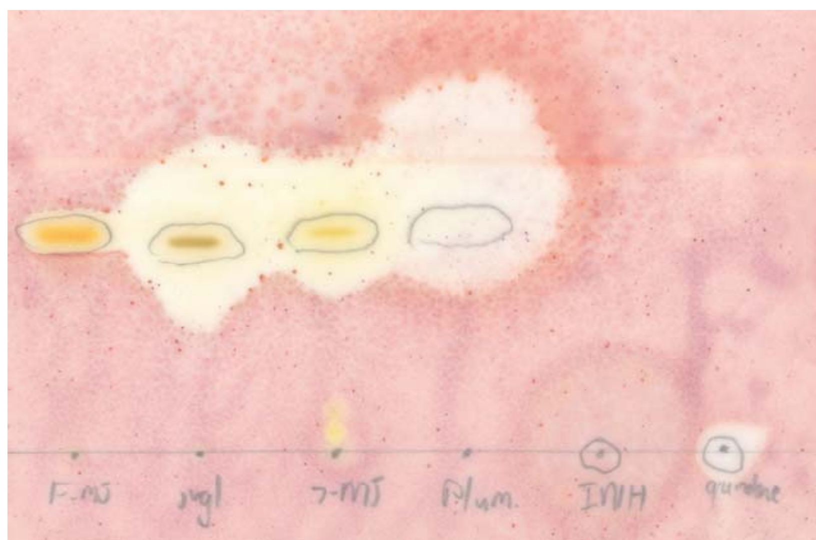


Fig. 8.5: Naphthoquinones developed on a TLC plate sprayed with *M. smegmatis*. Lanes 1-6 contain the fluorinated 7-methyljuglone, juglone, 7-methyljuglone, plumbagin, INH and moxifloxacin at amounts of 20 μg.

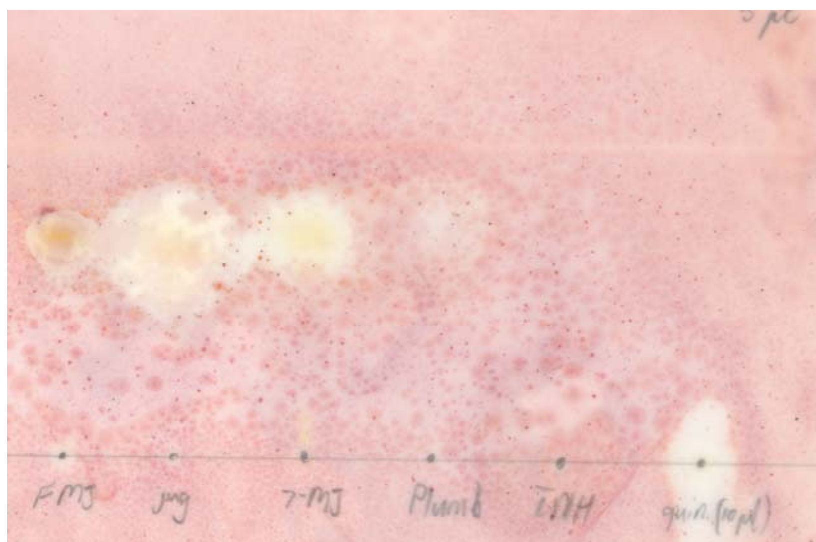


Fig. 8.6: Naphthoquinones developed on a TLC plate sprayed with *M. smegmatis*. Lanes 1-6 contain the fluorinated 7-methyljuglone, juglone, 7-methyljuglone, plumbagin, INH and moxifloxacin at amounts of 5 μg. The compounds are circled in pencil.

The fluorinated 7-methyljuglone has the highest log P value which would indicate that less diffusion through the 7H9 Middlebrook broth occurred. This might be the reason why it has a smaller zone of inhibition on the TLC plate. The difference between the polarity of INH and the naphthoquinones can also clearly be seen. The known drugs INH and moxifloxacin are more hydrophilic and stayed on the base line while the naphthoquinones are more hydrophobic.

8.3.2 *M. smegmatis* cultures

The compounds are stable for the first day after which they gradually break down or precipitate from solution. Therefore it was decided to stop the experiments 12 hours after the addition of the NQ's. The mass of the cells (wet and dry mass) are given in Table 8.2

Table 8.2: The mass of the *M. smegmatis* cells after 12h treatment with NQ's.

NQ	Mass (wet) mg	Mass (dry) mg
plumbagin	384	109
juglone	105	79
7-methyljuglone	181	74
fluorinated 7-methyljuglone	178	52
control	503	116

8.3.3 HPLC analysis

The HPLC chromatograms indicated that menaquinone was present in all the extracts except in the fluorinated 7- methyljuglone. This was clearly visible with the complete absence of the menaquinone peak. Figures 8.7-8.9 show the control, 7-methyljuglone and the fluorinated 7 - methyljuglone chromatograms. It is quite easy to recognise the apparent similarities between the chromatograms. There are four peaks with retention times between 3 and 7 minutes. These peaks are probably the shorter chained menaquinones. The ratio's and intensities between them are very similar and could therefore be used as indicator peaks.

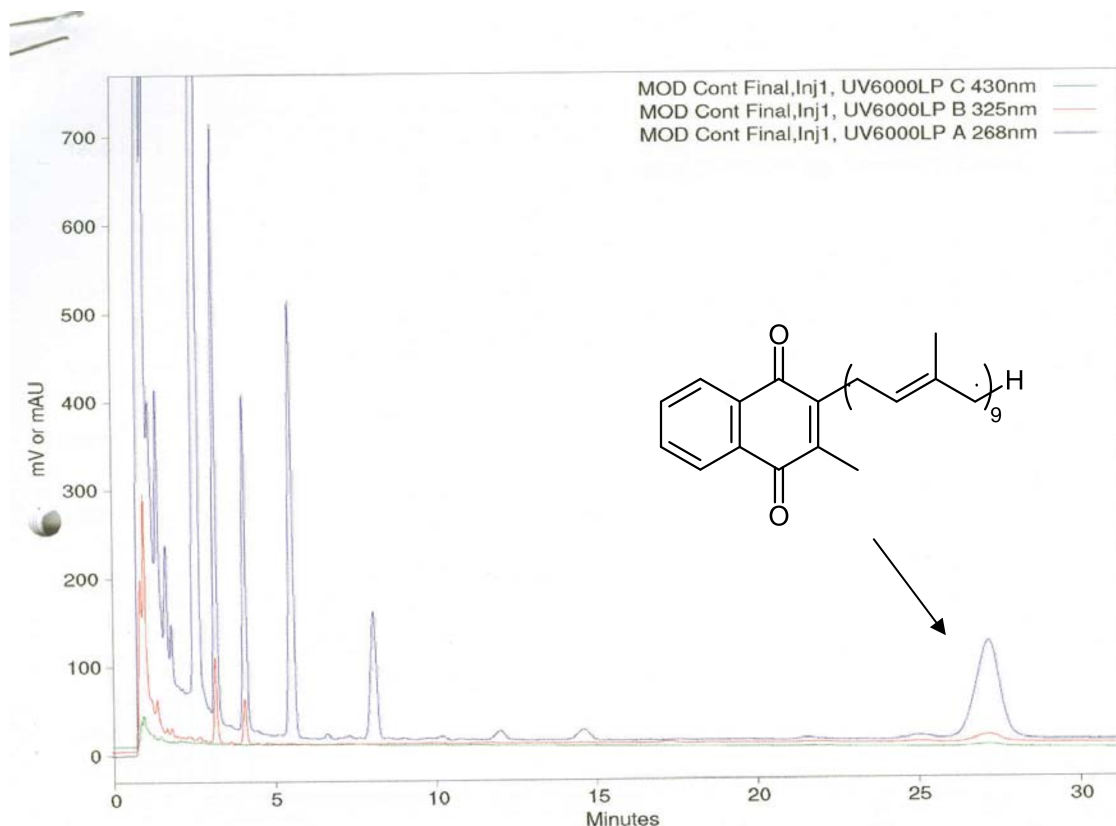


Fig. 8.7: HPLC chromatogram of the control sample indicating the presence of menaquinone at RT 27 min.

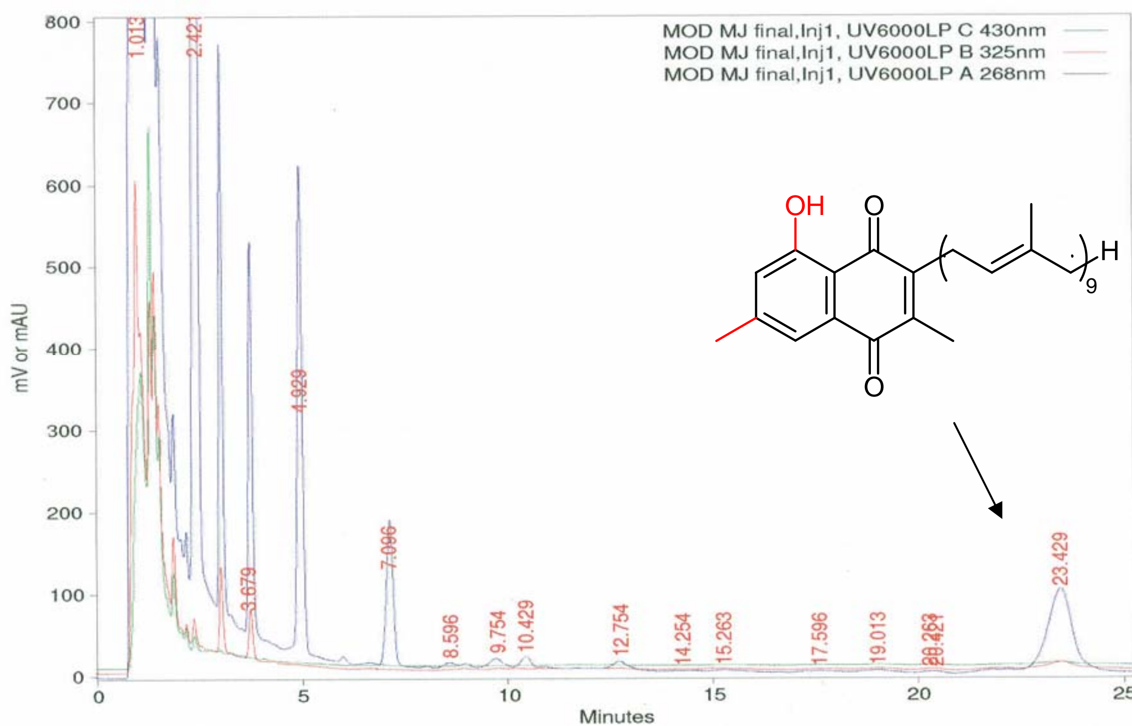


Fig. 8.8: Chromatogram indicating the presence of a menaquinone-like compound in the 7-methyljuglone sample. The retention time might indicate that this is a 7-methyljuglone with a terpenoid side chain.

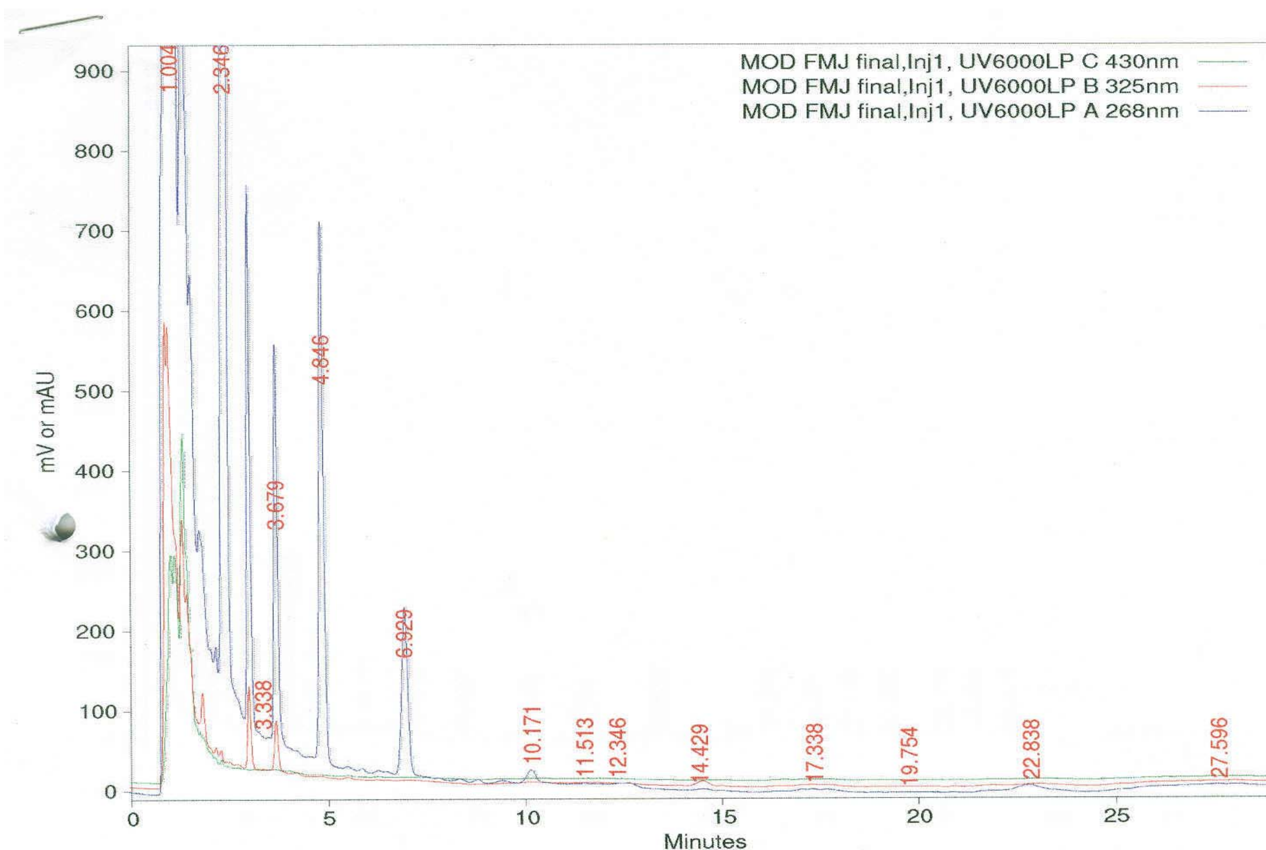


Fig. 8.9: Chromatogram of the fluorinated 7-methyljuglone sample indicating the absence of menaquinone.

8.4 Discussion and conclusions

The naphthoquinones tested on the TLC bioassay showed that the compounds are active against *M. smegmatis* and therefore it can be assumed that the mechanism of action could be the same as in *M. tuberculosis.* The yield of cells after sub-culturing for 5 days appears to be sufficient for one experiment. This must however be increased so that the experiments can be repeated in duplicate or triplicate. The mass of the cells give a good indication of the efficiency or potency of the compounds. Juglone stopped the growth of the cells the fastest while the fluorinated 7-methyljuglone and 7-methyljuglone**,** took slightly longer. The plumbagin sample appeared to be the most inefficient of the four NQ's tested. The HPLC results showed that menaquinone was completely inhibited by the fluorinated 7-methyljuglone. This will most probably be by binding to the men A or men G enzymes in the menaquinone biosynthetic pathway.

In the case of juglone and 7-methyljuglone, menaquinone still appeared to be present in the chromatogram. The altered retention time (2-3 minutes shorter RT) of the 7-methyljuglone treated sample might indicate that 7-methyljuglone could be incorporated into a menaquinone-like compound (which will have a reduced retention time due to its slightly more polar nature). This can also indicate that these compounds might bind to cytochrome bc complex or it can interfere with the electrons shuttled from the NADH dehydrogenase complex. This in fact will disrupt respiration with detrimental effect on the organism. This effect was also noticed with juglone and its influence on lettuce (Li *et al.,* 1993). This will influence the rate of respiration due to the altered redox potential that this new compound might have. The rate of menaquinone production and its half-life is however the key in understanding this system.

Currently a similar compound, moxifloxacin, in phase 3 clinical trials exhibits characteristics for this mechanism of action. This compound could interfere with the production of menaquinone at the *men* A biosynthetic step (Fig. 8.10). At the right pH the polyprenyl side chain can be attached to moxifloxacin, catalysed by men A. This hypothesis will be tested in future experiments which will also include 7-methyljuglone and the fluorinated 7-methyljuglone.

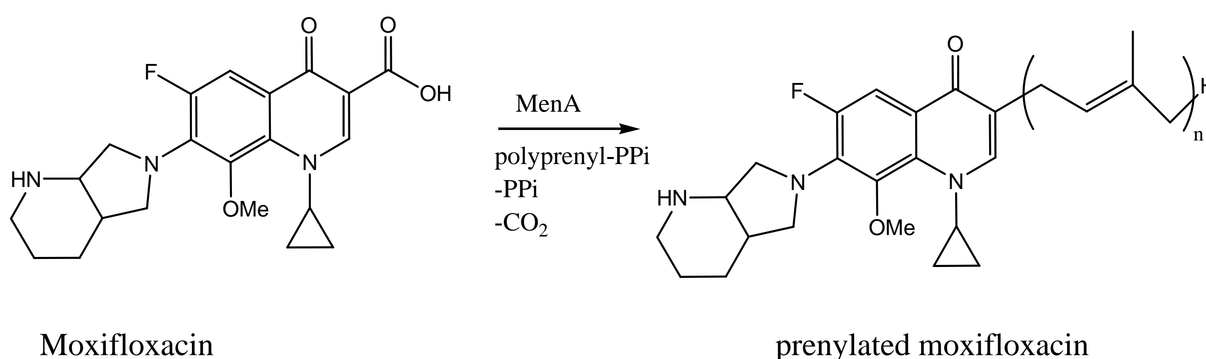


Fig. 8.10: Possible mode of action of moxifloxacin if it becomes prenylated through the enzymatic action of MenA, it might cause disruption of the electron transport chain

The structurally similar compound atovaquone (prescribed drug – mepron) is known to bind to the cytochrome *bc* complex in *Plasmodium falcipirum* (Fig. 8.11) (Yeo *et al*., 1997;

Srivastava *et al.*, 1999; Syafruddin *et al.,* 1999). During initial screening in our laboratory (unpublished results) of the structurally similar compound, neodiospyrin, the activity was found to be 60 ng/ml against *P. falcipirum* compared to 63 ng/ml for chloroquine (positive control). This would indicate that these compounds do indeed interfere with the electron transport chain of various organisms. The main challenge is therefore to change the structure of the compounds to such a degree as to make it species specific (and less toxic to people). In order to achieve this goal a large database of structurally similar compounds must be screened against various organisms.

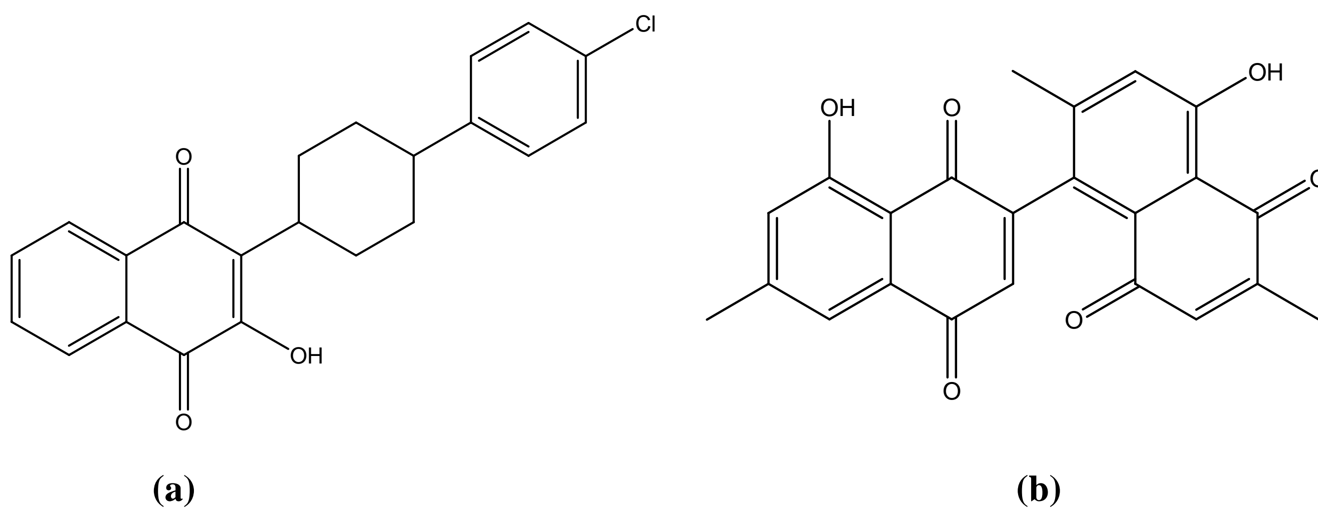


Fig. 8.11: The structural similarities between atovaquone (a) and neodiospyrin (b) indicates that the mode of action might be the same.

8.5 References

Bailly, C. (2000). Topoisomerase I poisons and suppressors as anticancer drugs. *Current Medicinal Chemistry*. 7(1), 39-58.

Begue, W. J. & Kline, R. M. (1972). Use of tetrazolium salts in bioautographic procedures. *Journal of Chromatography*. 64(1), 182-4.

Carter, F.L., Garlo, A.M., Stanley, J.B. (1978). Termicidal components of wood extracts: 7-methyljuglone from *Diospyros virginiana*. *Journal of Agriculture and food chemistry*. 26(4), 869-73.

Cushion, M. T., Collins, M., Hazra, B. & Kaneshiro, E. S. (2000). Effects of atovaquone and diospyrin-based drugs on the cellular ATP of *Pneumocystis carinii* f. sp. *carinii*. *Antimicrobial Agents and Chemotherapy*. 44(3), 713-719.

Eloff, J. N. (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants? *Journal of Ethnopharmacology*. 60(1), 1-8.

Kroppenstedt, R. M. (1982). Separation of bacterial menaquinones by HPLC using reverse phase (RS18) and a silver loaded ion exchanger as stationary phases. *Journal of Liquid Chromatography*. 5(12), 2359-67.

Lester, R. L. & Crane, F. L. (1959). Natural occurrence of coenzyme Q and related compounds. *Journal of Biological Chemistry*. 234, 2169-75.

Li, H. H., Nishimura, H., Koji, H. & Mizutani, J. (1993). Some physiological effects and the possible mechanism of action of juglone in plants. *Zasso Kenkyu*. 38(3), 214-22.

Srivastava, I. K., Morrisey, J. M., Darrouzet, E., Daldal, F. & Vaidya, A. B. (1999). Resistance mutations reveal the atovaquone-binding domain of cytochrome b in malaria parasites. *Molecular Microbiology*. 33(4), 704-711.

Steffen, K. & Peschel, H. (1975). Chemical constitution and antifungal activity of 1,4-naphthoquinones, their biosynthetic intermediates, and chemically related compounds. *Planta Medica.* 27(3), 201-12.

Syafruddin, D., Siregar, J. E. & Marzuki, S. (1999). Mutations in the cytochrome b gene of *Plasmodium berghei* conferring resistance to atovaquone. *Molecular and Biochemical Parasitology*. 104(2), 185-194.

Ting, C., Hsu, C., Hsu, H., Su, J., Chen, T., Tarn, W., Kuo, Y., Whang-Peng, J., Liu, L. F. & Hwang, J. (2003). Isodiospyrin as a novel human DNA topoisomerase I inhibitor. *Biochemical Pharmacology*. 66(10), 1981-1991.

Truglio, J. J., Theis, K., Feng, Y., Gajda, R., Machutta, C., Tonge, P. J. & Kisker, C. (2003). Crystal Structure of Mycobacterium tuberculosis MenB, a Key Enzyme in Vitamin K2 Biosynthesis. *Journal of Biological Chemistry*. 278(43), 42352-42360.

Wang, J., Burger, R. M. & Drlica, K. (1998). Role of superoxide in catalase-peroxidase-mediated isoniazid action against mycobacteria. *Antimicrobial Agents and Chemotherapy.* 42(3), 709-711.

Weinstein, E. A., Yano, T., Li, L., Avarbock, D., Avarbock, A., Helm, D., McColm, A. A., Duncan, K., Lonsdale, J.T. & Rubin, H. (2005). Inhibitors of type II NADH:menaquinone oxidoreductase represent a class of antitubercular drugs. *Proceedings of the National Academy of Sciences of the United States of America*. 102(12), 4548-4553.

Matsoso, L.G., Kana, B. D., Crellin, P. K., Lea-Smith, D. J., Pelosi, A., Powell, D., Dawes S. S., Rubin, H.., Coppel, R.L. & Mizrahi, V. (2005). Function of the cytochrome bc1-aa3 branch of the respiratory network in mycobacteria and network adaptation occurring in response to its disruption. *Journal of Bacteriology*. 187(18), 6300-8.

Yeo, A. E., Edstein, M. D., Shanks, G. D. & Rieckmann, K. H. (1997). Potentiation of the antimalarial activity of atovaquone by doxycycline against *Plasmodium falciparum in vitro*. *Parasitology research*. 83(5), 489-91.

Chapter 9
General discussion

It has been reported that the roots of *Euclea natalensis* A.DC. are used by indigenous people of southern Africa for various bacterial infections. The Zulu people used the roots for a purgative and for abdominal complaints and the root bark to treat tuberculosis (TB) related symptoms such as chest diseases, bronchitis, pleurisy and asthma. The Shangaan people apply the powdered root bark to skin lesions in leprosy and take it internally for ancylotomiasis while the Tonga people use the root for the relief of toothache and headache (Watt & Breyer-Brandwijk, 1962).

According to the Global TB Alliance's annual report, over 2 billion people carry the *Mycobacterium tuberculosis* bacterium. Millions of these infected people die each year. Most TB patients must complete 130 doses – up to eight tablets a day over a period of 6 months, while multi-drug resistant TB takes 2 years to treat. TB is also the leading killer of people with HIV-Aids, as current therapy cannot be combined easily with most HIV therapies. The incidence of TB is also on the increase with the last TB drug being commercialised over 30 years ago. In order to reverse the trend new anti-TB compounds must be found.

In total eleven naphthoquinones have been isolated from *E. natalensis,* while two of these compounds were isolated for the first time, during the authors M.Sc. studies (Van der Kooy, 2003). After finding a class of compounds that is active against a specific disease, the biochemical, chemical and biological aspects should be studied further. The aim of such a study is to better understand the biological role of these compounds in plants, humans (as medicine) and animals. During this thesis the chemical investigation included the organic synthesis of some of these naphthoquinones, as well as their stability and toxicity. In order to improve the potency of these compounds a structure–activity relationship has been conducted. From these preliminary findings the active and toxic sites could be identified. Future studies could focus on improving the potency with the addition of different side chains on carbon two and three. Due to the costs and time involved to synthesise these compounds additional funding and equipment need to be acquired. The semi-large scale production of the active compounds is needed for the various biological tests that have to be performed. Although the toxicity and stability tests made use of quantities in milligram, the *in vivo* mice work used

gram quantities. It is therefore essential to either isolate or synthesise enough material so that the various tests can be performed.

The compound, 7-methyljuglone**,** was synthesized by Musgrave & Skoyles (2001), in a two-step procedure. The overall yield of the two-step procedure (3 % calculated from the publication) is not satisfactory. During this thesis the synthesis was reinvestigated and improved to a one-step procedure with yields of 16 %. The synthesis of diospyrin**,** a dimer of 7-methyljuglone was published by Yoshida & Mori, *et al*. (2000). This synthetic route entails a 14 step sequence with yields of less than 4 %. Keeping in mind the costs, time and the experience needed for such a synthesis, it was decided that an alternative pathway was needed. During this thesis a new 3-step synthetic route with 7-methyljuglone epoxide as intermediate was investigated, to yield small quantities of diospyrin**.**

The stability of organic compounds in biological systems and bioassays was investigated in Chapter 5. This is a readily overlooked chemical test that bioactive compounds should be subjected to. It was found that the stability in almost all the bioassays used was unsatisfactory. This would indicate that the organic solvents used to dissolve hydrophobic compounds must be improved or the lead compounds need to be derivatised. The almost universal use of DMSO to dissolve hydrophobic compounds should be re-investigated.

The toxicity of the naphthoquinones was investigated in Chapter 6. The compounds proved to be toxic to vero cells, but these inflated results might be due to the high log P values of the compounds. A clear correlation could be seen between the log P values and the rate of precipitation in MEM. The toxicity of 7-methyljuglone was in addition also tested on *Musca domestica* to establish a possible mode of action of these compounds in diverse organisms. It proved to be non-toxic to the flies but did show some repellent properties.

Chapter 7 discussed the structure-activity relationship. This study was conducted in order to find the most potent and least toxic compound. Several structurally related compounds (isolated, synthesised or commercially obtained) have been tested for potency against TB to determine the active site (pharmacopore). In total thirteen compounds were tested for potency (with the use of the radiometric BACTEC bioassay) and for toxicity with the use of vero cells. In the pharmaceutical industry thousands of compounds are usually tested for potency with

the accuracy of the tests (structure-activity analysis) increasing with the increasing size of the data set.

It was found that the conjugate nucleophilic addition to α-β unsaturated carbonyl groups plays an important role, and it might be due to interaction via this mechanism with thiol groups (nucleophilic groups) on proteins that causes the toxicity of these compounds (McMurray, 1996: Lipinsky, personal communication). All the compounds with functional groups on both carbons (C2 and C3) have IC50 values of 128.5 μg/ml and higher. Only one of the three compounds, lapachol, showed good activity as well. This compound will be further derivatised in order to enhance its activity.

The mechanism of action of our lead compound and the biological activity in *M. smegmatis* indicated that there is a possible interference of the mycobacterial electron transport chain. These initial tests showed that 7-methyljuglone might be incorporated into a menaquinone-like compound, thereby inhibiting the flow of electrons or the rate of electron flow. The fluorinated 7-methyljuglone inhibited the production of menaquinone. The obtained results could be verified with the use of an LC-MS. This should give an indication if 7-methyljuglone was incorporated into a menaquinone-like compound (addition of 31 AMU). The use of *M. smegmatis* and *M. aureum* and the phase of bacterial growth (log phase etc.) and the influence this will have on potency should also be investigated. This can be done by administering the compounds during the different stages of growth. The bacteria could be cultured in different conditions especially aerobic and anaerobic conditions.

Future research could focus on finding additional structurally similar compounds from different sources in order to study the potency of these compounds on the electron transport chain. The structurally similar compound, atovaquone, is known to bind to the cytochrome bc complex in *Plasmodium falcipirum* (Srivastava *et al.*, 1999). During initial screening of neodiospyrin the activity was in the region of 60 ng/ml against *P. falcipirum*, which is comparable to chloroquine. This would indicate that these types of compounds do indeed interfere with the electron transport chain of various organisms. The main challenge is therefore to change the structure of the compounds to such a degree as to make it species specific. In order to achieve this goal a large database of structurally similar compounds must be screened against various organisms.

References

Global Alliance for TB Drug Development. (2005). pp1-3. Broad Street, 31st floor,New York, US.

McMurry, J. (1996). Organic chemistry. 4th ed. pp 1-3, 583, 882. Brookes/Cole Publishing, USA.

Musgrave, O.C. & Skoyles, D. (2001). Ebenaceae extractives. Part11. The synthesis of 7-methyljuglone. A re-examination. *Journal of the Chemical Society. Perkin Transactions*. 1 : 1318-1320.

Srivastava, I. K., Morrisey, J. M., Darrouzet, E., Daldal, F. & Vaidya, A. B. (1999). Resistance mutations reveal the atovaquone-binding domain of cytochrome b in malaria parasites. *Molecular Microbiology*. 33(4), 704-711.

Van Wyk, B. & Van Wyk, P. (1997). Field guide to trees of Southern Africa, pp 184-185. Struik, McKenzie street, Cape Town.

Van der Kooy, F. (2003). Characterisation, synthesis and antimycobacterial activity of naphthoquinones isolated from *Euclea natalensis*. Unpublished. M.Sc. dissertation. University of Pretoria. South Africa.

Watt, J.M. & Breyer-Brandwijk, M.G. (1962). The medicinal and poisonous plants of southern and eastern Africa. 2nd ed. p 390. Livingstone, Londen.

Yoshida, M. & Mori, K. (2000). Synthesis of diospyrin, a potential agent against Leishmaniasis and related parasitic protozoan diseases. *European Journal of Organic Chemistry*. 1313-1317.

Acknowledgements and Publications

I would like to thank the following people and institutions:

Prof. J.J.M. Meyer for his guidance, comments and suggestions during the course of this research project.

Mr Fanie de Meillon, Dr. Quenton Kritzinger, Rene Swart and the students in our group, for assistance and comments during the course of this research project.

Mrs. Patience Mativandla for the testing of the various compounds against TB.

Mrs. Brigitte Binneman and Dr Adriaan Basson for the vero cell toxicity testing.

Dr Kobus Venter from the MRC for the *in vitro* mice work.

The National Research Foundation and the University of Pretoria for financial support.

Publications from this thesis

1. Van der Kooy, F., Meyer, J.J.M. & Lall, N. (2006). Antimycobacterial activity and possible mode of action of newly isolated neodiospyrin and other naphthoquinones from *Euclea natalensis. South African Journal of Botany*. 72: 349-352.

2. Van der Kooy, F. & Meyer, J.J.M. (2006). Synthesis of the antimycobacterial naphthoquinone, 7-methyljuglone and its dimer, neodiospyrin. *South African Journal of Chemistry*. 59: 60-61.