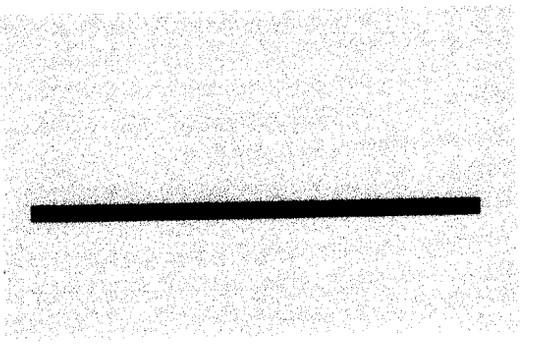
CHAPTER 5

ANTIBACTERIAL ACTIVITY OF *EUCLEA NATALENSIS*



Chapter 5

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Abstract

Water and acetone extracts of the roots of *Euclea natalensis* A.DC. were investigated for their *in vitro* antibacterial properties. The Gram-positive bacteria tested appeared to be more susceptible to the extracts than the Gram-negative bacteria. The water and acetone extracts inhibited the growth of *Bacillus cereus, B. pumilus, B. subtilis, Micrococcus kristinae* and *Staphylococcus aureus* at concentrations ranging between 0.1 and 6.0 mg/ml. The water extract did not exert any inhibitory action on Gram-negative bacteria while the acetone extract showed inhibitory activity at a concentration of 5.0 mg/ml against all the Gram-negative bacteria investigated. The antibacterial activity of the acetone extract of *E. natalensis* was also investigated by a direct bioassay on TLC plates against *S. aureus*.

5.1 Introduction

Euclea natalensis A.DC. a tree of Ebenaceae family, probably possesses medicinal properties because various plant parts are used in oral health care, for chest complains, bronchitis, pleurisy, chronic asthma, urinary tract infections, venereal diseases etc. by the indigenous people of South Africa (Figure 5.1). Roots of *E. natalensis* are boiled with thorns of *Phoenix reclinata, Maytenus heterophylla* and roots of *Capparis tomentosa* and tied to a sharp instrument, which is then stabbed, into the chest for pleurisy (Bryant 1966; Hutchings 1966). The Shangaans use the charred and powdered root as an application to the skin lesions in leprosy and internally for ancylostomiasis (Watt & Breyer-Brandwijk 1962; Bryant 1966). In one study samples of fresh root were tested against *Streptococcus mutans* and human saliva and periodontal pocket isolates and it was found that aerobic as well as anaerobic bacterial growth was suppressed in all instances (Stander & Van Wyk, 1991).

Other species of *Euclea* have been cited in the literature as being used for medication or for containing biologically active compounds. The antifungal activity of some 1,4-naphthoquinones including lawsone, juglone, and 7-methyljuglone isolated from *E. lanceolata*, *E. undulata* and *E. multiflora* has been reported (Watt & Breyer Brandwijk 1962; Steffen & Peschel 1975). In addition to antifungal action, juglone is a well known haemostatic agent (Stecher 1968). Naphthoquinones such as mamegakinone, 7-methyljuglone have also been reported to be antibacterial against *Neisseria gonorrhoeae, Shigella dysenteriae* and *S. flexnerii* (Khan *et al.* 1978). A preliminary antibacterial assay showed that the petroleum ether and chloroform extracts of *E. natalensis* root bark gave an inhibitory zone of 15 mm, at an extract concentration of 0.3 mg/ml against *Staphylococcus aureus* (Khan *et al.* 1978).

Plants have always been a common source of medicaments, either in the form of traditional preparations or as pure active principles. One of the major approaches in

developing new drugs from plants is to examine the uses claimed in traditional use. Many of the reports on the pharmacological testing of crude plant extracts have been published by investigators working in laboratories in developing countries (Fransworth *et al.* 1985). Since the root of *E. natalensis* is extensively used in various infectious diseases by traditional practitioners the current investigation was undertaken. The antibacterial properties of water and acetone extracts of the roots of *E. natalensis* against 11 bacterial species are described.

Bioautography, as a method to localise antibacterial activity on a chromatogram, has found widespread application in the search for new antibiotics. A suspension of a microorganism in a suitable broth is applied to a developed thin layer chromatography (TLC) plate and incubation in a humid atmosphere which permits growth of the bacteria. Zones of inhibition are then visualised by a dehydrogenase activity-detecting reagent, i.e. a tetrazolium salt (Hamburger & Cordell 1987). The antibacterial activity of acetone extract was also investigated by direct bioassay on TLC plates against *S. aureus* in this study.

Figure 5.1 Roots of *E. natalensis*

5.2 Materials and methods

5.2.1 Plant material

Roots of *E. natalensis* were collected from Mputaland, a region in the KwaZulu-Natal province of South Africa. A voucher specimen (N.L. 22) was deposited and identified at the H.G.W.J. Schweickerdt Herbarium (PRU), Pretoria.

5.2.2 Preparation of plant extracts

The acetone extract of *E. natalensis* was prepared by stirring 40 g of powdered root in 2 l of acetone over two days under reflux. The extract was filtered and concentrated to dryness at reduced pressure. The resultant residue was later dissolved in acetone to a concentration of 100 mg/ml. The water extract was prepared by boiling 20 g of powdered root in 500 ml distilled water for 30 min under reflux. The extract was filtered and concentrated to dryness under reduced pressure. The residue was later dissolved in distilled water to a concentration of 100 mg/ml.

5.2.3 Bacteria

Eleven bacterial species (Table 5.1) were collected from the Department of Microbiology and Plant Pathology, University of Pretoria. Each organism was maintained on nutrient agar slant and was recovered for testing by growth in nutrient broth (No. 2, Biolab) for 48 h at 37°C. Before streaking, each culture was diluted 1:10 with fresh sterile nutrient broth.

5.2.4 Antibacterial testing

The minimal inhibitory concentration (MIC) of the extracts was determined by incorporating various amounts (0.1-6.0 mg/ml) of the extract into Petri dishes containing the culture media. Plant extracts were introduced aseptically into sterile Petri dishes by filtering through sterile 0.22 µm syringe fitted filters and then added to autoclaved nutrient agar (Biolab). Before congealing, 5 ml of nutrient agar medium containing the plant extract was added aseptically to each Petri dish and swirled carefully until the agar began to set. The organisms were streaked in radial patterns on agar plates containing plant extracts, incubated at 37^0C and observed after 24 h (Mitscher *et al.* 1972). Water and acetone extracts were tested at 6.0, 5.0, 1.0, 0.5 and 0.1 mg/ml. Two blank plates containing only nutrient agar and two containing nutrient agar and 1% acetone without the plant extracts served as controls. The MIC was regarded as the lowest concentration of the extracts that did not permit any visible growth. Each treatment was replicated three times.

Direct bioassays on TLC plates were done by applying 20 µl of the acetone extract (20 mg/ml) to silica gel 60 plates (Merck). The plates were developed in chloroform-hexane (1:1) and carefully dried over cold air for complete removal of the solvents. A 48 h old *S. aureus* culture in nutrient broth was centrifuged at 1090 x g for 20 min, the supernatant was discarded and the pellet resuspended in fresh nutrient to an absorbance of 0.84 at 560 nm. A fine spray was used to spray the bacterial suspension onto the TLC plates. These plates were then dried until they appeared translucent and incubated at 25^0C for 48 h under humid conditions. After incubation, the plates were sprayed with an aqueous solution of 2 mg/ml *p*-iodonitrotetrazolium violet. The plates were then reincubated at 25^0C for 24 h (Lund & Lyon 1975). Metabolically active bacteria convert the tetrazolium salt into the corresponding intensely coloured formazan and the antibacterial compounds appear as clear spots against a coloured background (Hamburger & Cordell 1987). Each test was replicated three times.

5.3 Results and Discussion

The antibacterial assays of the root extracts of *E. natalensis*, showed that the acetone extract was significantly active against the Gram-positive organisms tested (Table 5.1). The extract inhibited the growth of *Micrococcus kristinae* at 0.1 mg/ml, which was the highest dilution used in this study. In addition, all the Gram-negative organisms were inhibited at a concentration of 5.0 mg/ml. The water extract was found to be more effective against *Bacillus cereus, B. pumilus* and *M. kristinae* than against *B. subtilus* and *S. aureus*. However, the water extract did not inhibit the growth of any of the tested Gram-negative bacterial species.

Plants possess potentially therapeutic agents that provide an impetus for further research (Recio & Rios 1989). Testing microorganisms for their susceptibility to plant extracts is a common place laboratory procedure that serves as an important aid to chemotherapeutic intervention in cases of infection. Several plants have been reported in the literature for their antibacterial activity similar to the results obtained in this study. Extracts of *Moneses uniflora* exhibited good activity against a number of Gram-positive and Gram-negative bacteria (McCutcheon *et al.* 1992). It has been reported earlier that the chloroform extract of *Zizyphi fructus* had inhibitory activity against insoluble glucan formation by glucosyltransferase from the cryogenic bacterium, *Streptococcus mutans* (Kohda *et al.* 1985). Roots of *Plumbago auriculata* and *P. zeylanica* showed significant antibacterial activity against *Klebsiella aerogenes, S. aureus, B. pumilus* and *B. cereus* (Van der Vijver & Lotter 1971). The results of this study demonstrated that water and acetone extracts of *E. natalensis* produced *in vitro* antibacterial activity against some medically important microorganisms. The bacteria inhibited in our study have been associated with infections, which the local inhabitants treat with *E. natalensis* extracts. Also, the fact that the extract inhibited *S. aureus* and *K. pneumoniae* which are associated with respiratory tract infections and chest complaints, provides some scientific rationale for the use of the extracts for

bronchitis, pleurisy, chronic asthma etc. Our study confirms the previous reports on anti-staphylococcal activity of this plant (Khan *et al.* 1978).

Table 5.1 Antibacterial activity of aqueous and acetone extracts of the roots of *E. natalensis*

Bacterial species	Gram +/-	MIC[a] (mg/ml)	
		Aqueous	Acetone
Bacillus cereus	+	5.0	0.5
B. pumilus	+	5.0	0.5
B. subtilis	+	6.0	0.5
Micrococcus kristinae	+	5.0	0.1
Staphylococcus aureus	+	6.0	0.5
Enterobacter cloacae	-	na[b]	5.0
E. aerogenes	-	na	5.0
Escherichia coli	-	na	5.0
Klebsiella pneumoniae	-	na	5.0
Pseudomonas aeruginosa	-	na	5.0
Serratia marcescens	-	na	5.0

[a]minimal inhibitory concentration.
[b]not active.

The Gram-positive bacteria appeared to be more susceptible to the inhibitory effect of the extracts than the Gram-negative organisms. Similar observations were made by Kuhnt *et al.* (1994), Meyer and Afolayan (1995) and Saxena *et al.* (1996) while studying the antibacterial activity of *Hyptis verticillata, Helichrysum aureonitens* and *M. uniflora*, respectively. The weak activity shown by the acetone extract against

the Gram-negative bacteria, could be due to the presence of compounds in the extract possessing lipophilic characteristics as suggested by Werner *et al.* (1979).

Six zones of bacterial growth inhibition could be seen on TLC plates sprayed with *S. aureus*. Previous phytochemical analyses have shown the presence of biologically active constituents such as naphthoquinones, triterpenoids, terpenoids etc. in *Euclea* spp. (Watt & Breyer-Brandwijk 1962; Ferreira *et al.* 1977). These compounds may have been responsible for the antibacterial activities observed in the present study.

5.4 Conclusion

The *in vitro* results obtained in this study probably justify the traditional use of *E. natalensis* for various ailments. The Gram-positive bacteria tested appeared to be more susceptible to the extracts than the Gram-negative bacteria. Six zones of bacterial growth inhibition was seen on TLC plates sprayed with *S. aureus* in a direct bioassay of the acetone extract of *E. natalensis*.

5.5 References

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CHAPTER 6

ANTIBACTERIAL ACTIVITY OF DIOSPYRIN ISOLATED FROM *EUCLEA NATALENSIS*

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Abstract

The binaphthoquinoid, diospyrin, was isolated from *Euclea natalensis* A. DC., and evaluated for its activity against drug-sensitive and resistant strains of *Mycobacterium tuberculosis* and other bacteria. The minimal inhibitory concentration (MIC), of diospyrin was found to be 100 µg/ml for all the *M. tuberculosis* strains. It was also tested against five Gram-positive and six Gram-negative bacteria. The MIC was also 100 µg/ml for Gram-positive organisms, *Bacillus cereus, B. pumilus, B. subtilis, Micrococcus kristinae* and *Staphylococcus aureus.* No inhibitory effect of the compound was observed on any Gram-negative bacteria at the highest concentration (100 µg/ml) tested.

6.1 Introduction

The resurgence of tuberculosis (TB) as a major disease in many parts of the world is prompting the search for novel compounds active against the causative organism, *Mycobacterium tuberculosis*. Out of 20 medicinal plants investigated in our laboratory, *E. natalensis* exhibited the best activity against drug-sensitive and resistant strains of *M. tuberculosis* (Lall & Meyer 1999).

The large subtropical genus *Euclea* is well known as a source of naphthoquinones. Monomers, complex dimers and trimers such as 7-methyljuglone, diospyrin and mamegakinone have been isolated from them (Watt & Breyer-Brandwijk 1962; Bryant 1966; Khan *et al.*1978; Siddiqi *et al.* 1981; Hazra *et al.* 1984; Pujol 1990) but nothing has been reported concerning their antimycobacterial activity. The inhibitory effect of the crude extract of *E. natalensis* against a drug-sensitive and two drug-resistant strains of *M. tuberculosis* have been investigated (Lall & Meyer 1999). We now report, on the isolation and identification of the active principle from *E. natalensis* and its inhibitory effect against the drug-sensitive and drug-resistant strains of *M. tuberculosis*.

Antibacterial activity has been attributed to the occurrence of naphthoquinones in *Diospyros* and *Euclea* species (Waterman & Mbi 1979). Antibacterial and antifungal screening of diospyrin, isolated from *E. natalensis*, has been done previously. It was found that diospyrin, inhibited the growth of *Bacillus cereus* at a concentration of 0.3 mg/ml and no inhibitory effect was observed against *Staphylococcus aureus* at this concentration (Khan *et al.* 1978). To determine the minimal inhibitory concentration (MIC) of diospyrin for several other bacterial species, the activity of the compound was investigated against five Gram-positive and six Gram-negative bacteria in this study. Diospyrin was also evaluated by a direct bioautographic assay on TLC plates against *S. aureus*.

6.2 Materials and Methods

6.2.1 Extraction, isolation and purification of the active compound

The dried powdered root (100g) of *E. natalensis* was left to macerate in acetone (1l) at room temperature for two days. After filtration, the solvent was concentrated under reduced pressure to give a crude extract (4.2 g, 4.2% of dry weight). A direct bioassay on *S. aureus* of the crude acetone extract on thin layer chromatography (TLC) plates, showed that a compound of Rf 0.30, had antibacterial activity. In order to purify this antibacterial compound the crude extract of the plant (4.2 g) was dissolved in $CHCl_3$ and fractionated on silica gel 60 by column chromatography, using $CHCl_3$ as eluent. This achieved a preliminary separation from unwanted tars and macromolecules, which are unable to pass through the silica column. The bioactive antibacterial fractions also showed activity against *M. tuberculosis* and were then subjected to Sephadex LH-20 column chromatography, using ethanol as eluent. The compound was further purified by HPLC utilising an analytical Phenomenex reverse phase 250x4.60 mm column, at a flow rate of 1.0 ml/min, temp. 40^0C and a wavelength of 206 nm. An ethanol:water (50:50) solution was employed as mobile phase. ^{1}H-NMR, and ^{13}C-NMR, were obtained at 300 MHz on a Bruker ARX 300 NMR spectrometer using $CDCl_3$ as solvent with tetramethylsaline (TMS) as internal standard.

6.2.2 Bioassay on *M. tuberculosis*

The radiometric respiratory technique with the BACTEC was used for susceptibility testing of *M. tuberculosis* as described earlier (Middlebrook *et al.* 1977; Siddiqi *et al.* 1981). Bacterial cultures utilized in this study were grown from specimens received from the Medical Research Council (MRC) in Pretoria. A sensitive strain of *M. tuberculosis*, H37Rv reference strain and six other multidrug-resistant strains (MDR strains) were used in the screening procedure (Table 6.1).

Diospyrin was dissolved at 10 mg/ml in 1% DMSO and stored at -4^{0}C until used. Subsequent dilutions were done in DMSO and added to 4ml of BACTEC 12B broth to achieve the desired final concentrations of 100, 50 and 10 µg/ml together with PANTA (Becton Dickinson & Company), an antimicrobial supplement. BACTEC drug susceptibility testing was also done for the two primary TB-drugs, streptomycin and ethambutol at concentrations of 6 µg/ml and 7.5 µg/ml respectively against H37Rv strain.

A homogenized culture (0.1 ml) of all the strains of *M. tuberculosis*, yielding 1x10^4 to 1x10^5 colony forming units/ml (CFU/ml), were inoculated in the vials containing the compound as well as in the control vials (Heifets *et al.* 1985). Two plant extract-free vials were used as controls: 1 vial was inoculated in the same way as the vials containing the compound, and the other was inoculated with a 1:100 dilution of the inoculum (1:100 control) to produce an initial concentration representing 1% of the bacterial population (1x10^2 to 1x10^3 CFU/ml) found in the vials containing diospyrin. The MIC was defined as the lowest concentration of the compound that inhibited more than 99% of the bacterial population.

Inoculated bottles were incubated at 38^{0}C and each bottle was assayed everyday at about the same hour until cumulative results were interpretable. The difference in the GI values of the last two days is designated as ΔGI. The GI reading of the vials containing the plant extract was compared with the control vial, containing a 1:100 dilution of the inoculum. Readings were taken until the control vials containing a 100 times lower dilution of the inoculum, than the vials with plant extract, reached a GI of 30 or more. If the ΔGI value of the vial containing the plant extract was less than the control, the population was reported to be susceptible to the extract.

6.2.3 Antibacterial testing

Diospyrin was also tested for inhibitory activity against five Gram-positive and six Gram-negative bacterial strains. Gram-positive bacterial species analysed were *Bacillus cereus, B. pumilus, B. subtilis, Micrococcus kristinae, Staphylococcus aureus*, and the Gram-negative bacterial species were *Enterobacter cloaccae, E. aerogenes, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Serratia marcescens*. Bacterial species were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria.

The minimal inhibitory concentration (MIC) of diospyrin was determined by incorporating various amounts (100.0, 50.0, 1.0 and 0.5 µg/ml) of the solution containing the compound, into petri dishes containing the culture media as done previously by Meyer and Dilika (1996). The organisms were streaked in radial patterns on agar plates containing plant extracts, incubated at 37^0C and observed after 24h (Mitscher *et al.* 1972). Two blank plates containing only nutrient agar and two containing nutrient agar and 1% acetone without the extracts, served as controls to ensure the validity of the test. In addition plates containing streptomycin sulfate at concentrations of 200, 100, 50 and 10 µg/ml served as positive drug controls. The MIC was regarded as the lowest concentration of the extracts that did not permit any visible growth when compared with that of the controls.

Direct bioassays of the extract and diospyrin against *S. aureus* were done by applying 20 µl extract or diospyrin dissolved in acetone (20 mg/ml) to silica gel 60 plates (Merck) according to the procedures described previously by Lund and Lyon (1975). These TLC plates were then developed with chloroform-hexane (1:1) and a fine spray was used to spray a bacterial suspension of *S. aureus* onto the TLC plates. The plates were then incubated for 48 h in humid conditions. After incubation, the plates were sprayed with an aqueous solution of 2 mg/ml p-iodonitrotetrazolium violet. Each test was replicated three times.

6.3 Results and Discussion

6.3.1 Identification of the isolated compound

The UV spectrum of the compound with maxima at 254 and 430 nm and the bathochromic shift on the addition of alkali was typical of a naphthoquinone (Figure 6.1).

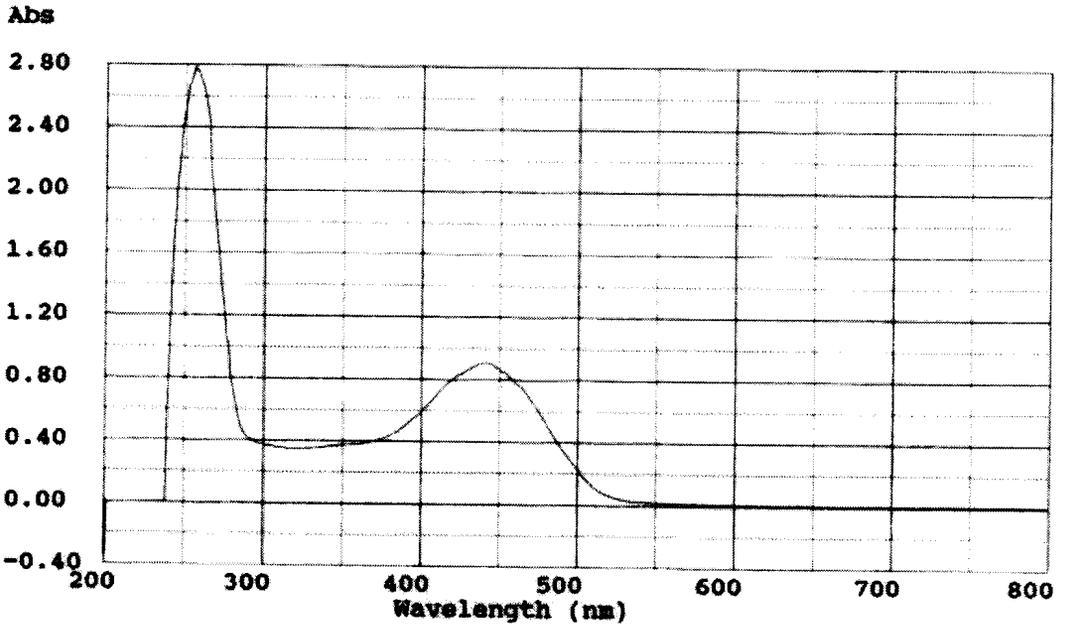

Figure 6.1 UV spectrum of diospyrin isolated from *E. natalensis*

The compound was identified as the binaphthoquinone, diospyrin, by comparison of its ^{1}H-NMR and ^{13}C-NMR spectral data with published spectra (Hazra *et al.* 1984). The ^{1}H-NMR spectrum exhibited signals at δ 2.28 and δ 2.43 from the two methyl groups, at δ 11.86 and δ 12.11 from the two hydroxyl groups, one proton singlets at δ 6.89, 7.10, 7.48, 7.54 and a doublet at δ 6.93 (Figure 6.2). The ^{13}C-NMR spectrum shows twenty two lines corresponding to its molecular formula $C_{22}H_{14}O_6$. The signals of the unsubstituted carbons are more intense and can be distinguished from the weak lines of the unsubstituted ones (Figure 6.3). The identification was also confirmed by direct comparison with authentic samples on TLC. Diospyrin has also been isolated from other species of *Euclea* such as *E. crispa, E. divinorum* and *E. schimperi* (Van der Vijver & Gerritsma 1974), and from the *Diospyros* species, *D. mannii* (Jeffreys *et al.* 1983), *D. chamaethamus* (Mitscher *et al.* 1972) and *D. montana* (Hazra *et al.* 1984) (Figure 6.4).

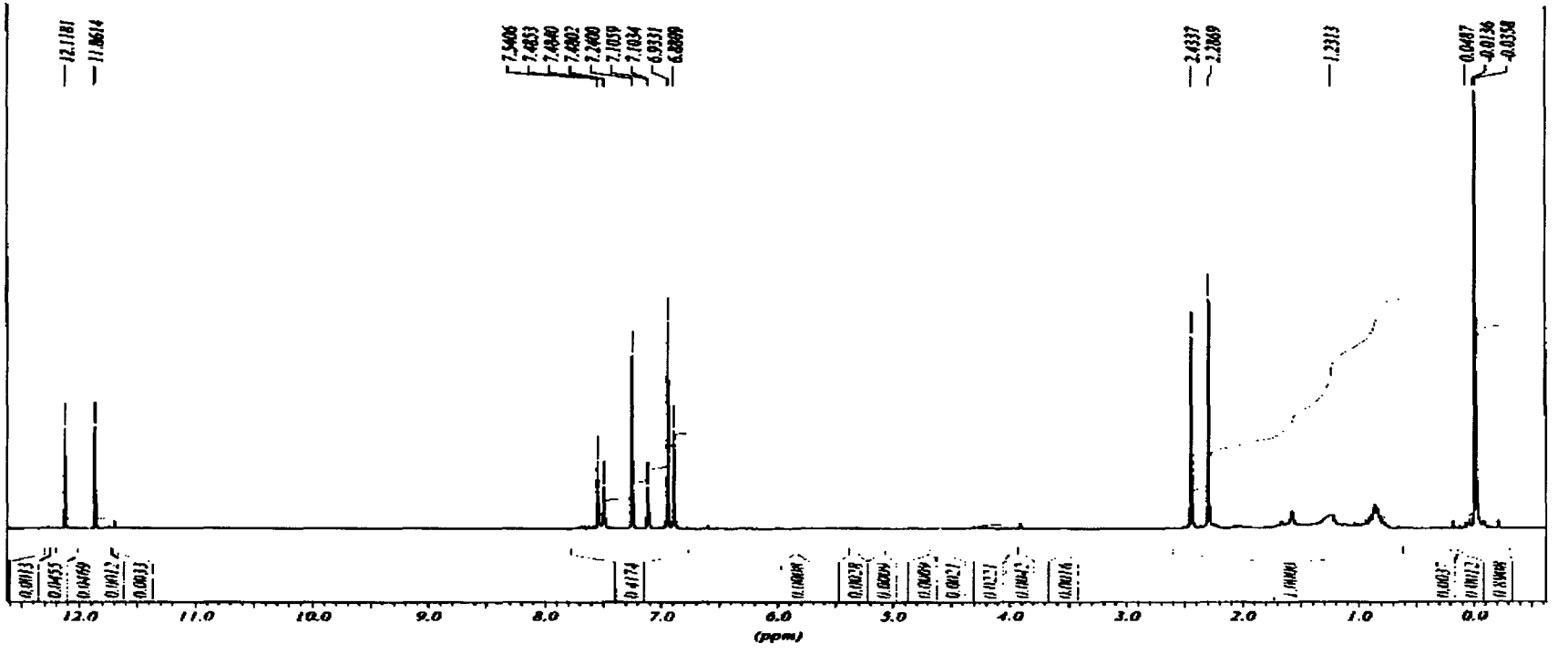

Figure 6.2 ^{1}H-NMR spectrum of diospyrin isolated from *E. natalensis*

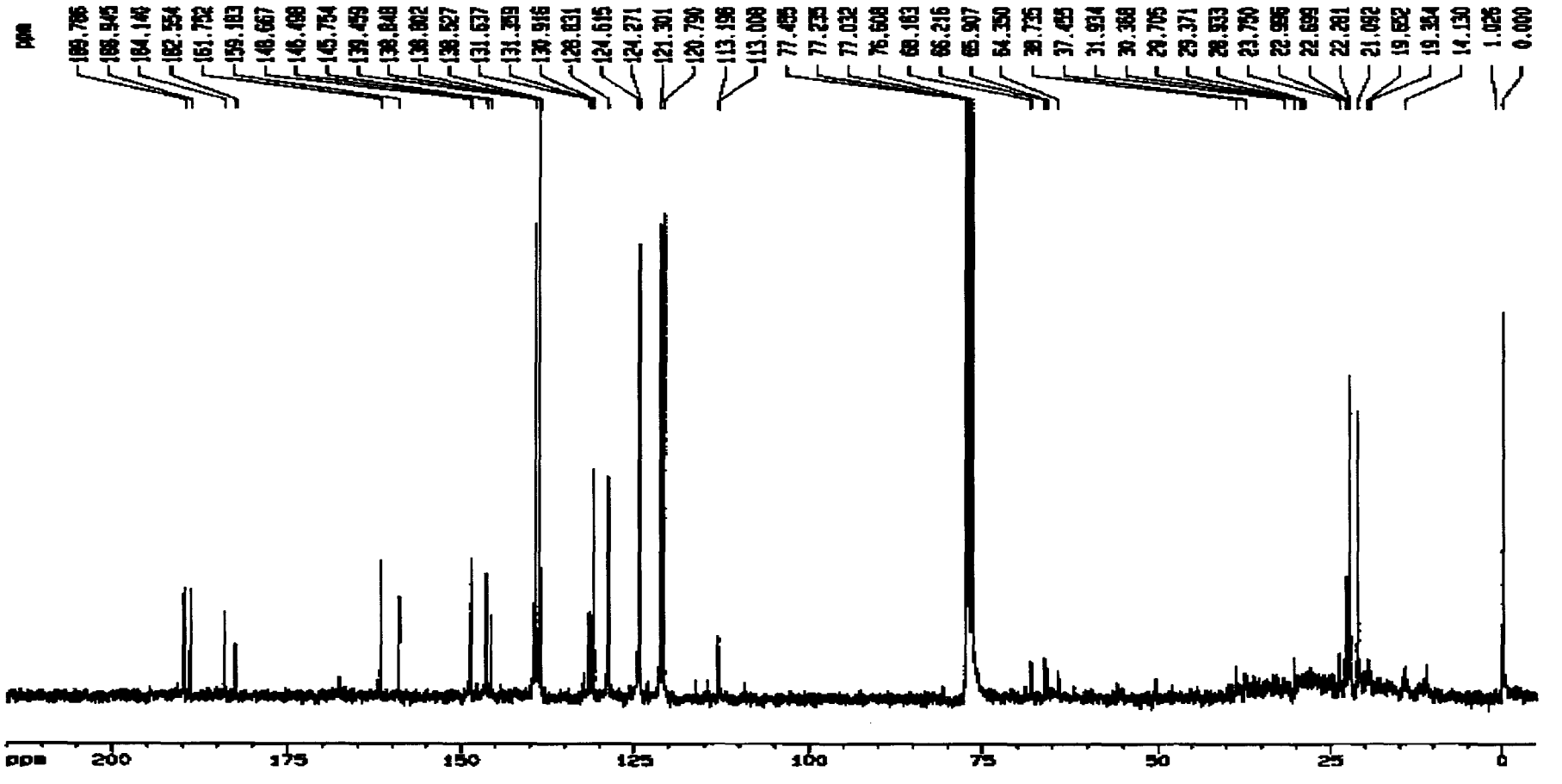

Figure 6.3 ^{13}C-NMR spectrum of diospyrin isolated from *E. natalensis*

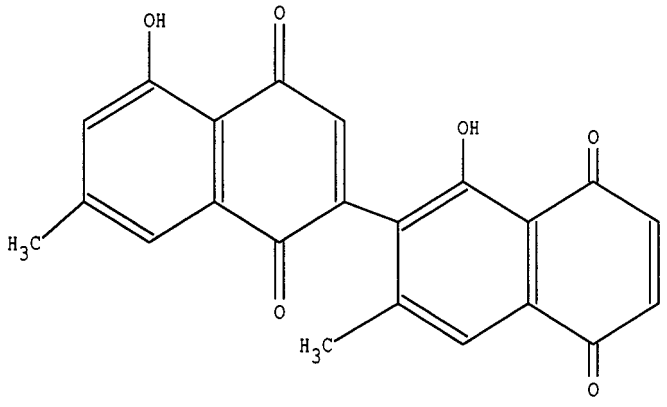

Figure 6.4 Chemical structure of diospyrin

6.3.2 Effect of diospyrin on *M. tuberculosis*

Results were interpreted on day 6 or 7 when the control vials containing the 1:100 dilution of the inoculum reached a GI value of 30 or more (Table 6.1). The MIC of diospyrin was found to be 0.1 mg/ml against the H37Rv strain as well as against the two to seven drug resistant strains. However, the ΔGI values of the vials containing diospyrin at 0.05 mg/ml concentration level, for the two and three drug-resistant strains were found to be 20 ± 3.60 and 13 ± 6.55 respectively. This indicated that these strains are partially susceptible to the compound. The ΔGI values of the vials containing streptomycin and ethambutol was found to be 2 ± 1.41 and 5 ± 2.12 respectively.

Over the past decade there has been a proliferation in literature on the antimycobacterial properties of plant extracts. The MIC values of compounds isolated from several other plants were found to be generally higher than that of diospyrin. It has been reported that he alkaloids isolated from *Galipea*

Table 6.1 Effect of diospyrin on the growth of the sensitive strain (H37Rv) and resistant strains of *Mycobacterium tuberculosis* as determined by the radiometric method

Mycobacterium tuberculosis strains	Lab reference no.	MIC (µg/ml)	ΔGI[a] values of plant extracts	ΔGI values of the control vial
H37 sensitive strain	ATCC27294	100	-1 ± 1.41	20 ± 4.24
MDR strains (strains resistant to the following drugs)[b]				
I and R	CCK028469V	100	3.5 ± 0.70	25 ± 7.07
S, I and E1	C9	100	4 ± 2.12	29 ± 1.41
S, I, R and E1	C84	100	5 ± 2.82	25 ± 2.82
I, S, R, T1 and C	CGT1296429	100	10 ± 1.41	22.5 ± 3.53
I, R, E2, T1, T2 and O	CCK070370H	100	9 ± 2.82	30 ± 1.0
I, S, E1, E2, K, R, and T1	CGT1330497	100	13.5 ±3.2	28 ± 3.1

[a]ΔGI values are means ± standard deviation.

[b]Drugs: I-isoniazid; R-rifampin; S-streptomycin; E1-ethambutol; E2-ethionamide; T1-thiacetazone; T2-terizidone; C-cyclocerine; O-ofloxacin; K-kanamycin.

officinalis had inhibitory activity against ten strains of *M. tuberculosis* at concentrations from 0.7 to 0.01 mg/ml (Houghton *et al.* 1999). Hypargenin F, isolated from the roots of *Salvia hypargeia* exhibited activity against *M. tuberculosis* (H37Rv) at 0.25 mg/ml (Ulubelen *et al.* 1997). The concentration required for the principal antimicrobial component of garlic oil, allicin, to inhibit six strains of *M. tuberculosis*, ranged from 1.34 mg/ml to 2.68 mg/ml (Delaha & Garagusi 1985). The MIC of ambroxol, a semi-synthetic derivative of vasicine from the Indian shrub Adhatoda vasica, was found to be 50 mg/ml for ten clinical isolates of *M. tuberculosis* (Grange & Snell 1996).

The quinones exhibiting inhibitory activity against *M. tuberculosis* were found to be multiorthoquinone and 12-demethylmultiorthoquinone isolated from *Salvia multicaulis* (Ulubelen *et al.* 1997). According to previous reports, among the triterpenes isolated from *Borrichia frutescens*, the presence of a C-3 keto in (24R)-24,25-epoxycycloartan-3-one and/or a β-hydroxy group in (3∝H, 24R)-24,25-epoxycycloartan-3-ol seems to play a major role in the *in vitro* antituberculosis activity (MIC 64-128μg/ml) (Cantrell *et al.* 1996) (Figure 6.5). However, it requires further verification by testing structurally related naphthoquinones to determine the essential active regions necessary for significant antituberculosis activity.

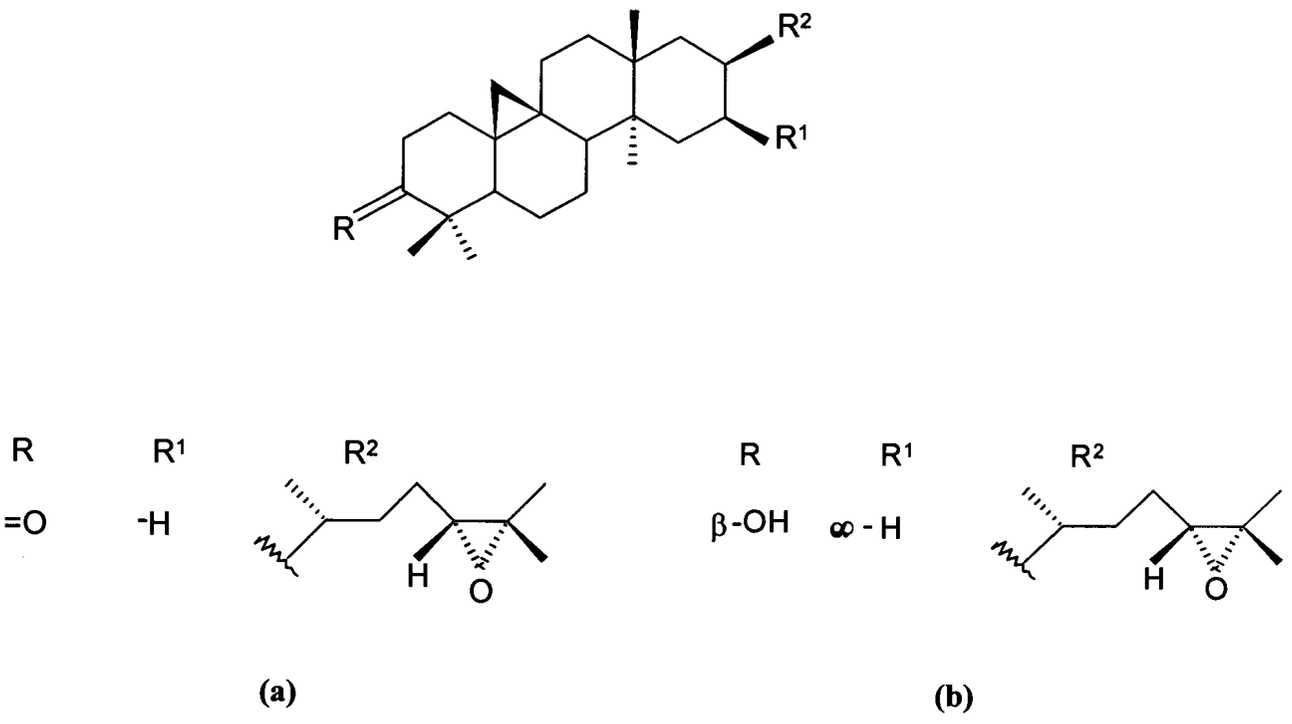

Figure 6.5 Triterpenes: (a) (24R)-24,25-epoxycycloartan-3-one. (b) (3∝H,24R)-24,25-epoxycycloartan-3-ol isolated from *Borrichia frutescens*

6.3.3 Effect of diospyrin on bacterial species

The MIC of diospyrin was found to be 0.1 mg/ml against all the Gram-positive bacteria tested. Diospyrin did not inhibit the growth of any of the tested Gram-negative species. The reference antibiotic, streptomycin sulfate inhibited the growth of all the bacterial species tested in this study at 10 µg/ml except, *Pseudomonas aeruginosa* and *Serratia marcescens* which were inhibited at 50 µg/ml and 100 µg/ml respectively. Direct bioautography on TLC plates has shown that diospyrin is one of

the major antibacterial compounds in the acetone extract from *E. natalensis* (Figure 6.6).

The antifungal, antibacterial and termite resistant properties of *Diospyros* species have all been attributed to the occurrence of naphthoquinones (Khan *et al.* 1978; Hazra *et al.* 1984). It has been stated before that the root of *E. natalensis* contains naphthoquinones, which are bactericidal (Stander & Van Wyk 1991). Naphthoquinones isolated or obtained synthetically, have been proved to possess antimicrobial activity against bacteria, specifically against Gram-positive bacteria, yeasts, and filamentous fungi (Berdy *et al.* 1980). Out of 22 naphthoquinones (diospyrin was not tested), Baker *et al.* (1990) found that 15 exhibited antibiotic activity against *S. aureus*, and 12 were active against *Streptococcus pyrogenes* but none were active at the highest concentration tested (128 µg/ml) against *E. coli, K. pneumonia, Salmonella typhi, Proteus vulgaris, Serratia marcescens,* or *Pseudomonas aeruginosa*. A quinone antibiotic guacin, showed a good inhibitory effect against Gram-positive bacteria including multidrug-resistant strains at concentrations ranging between 0.02 and 0.09 µg/ml. Werner *et al.* (1979), adds that the relatively strong inhibitory effect of these compounds against Gram-positive bacteria in contrast to the Gram-negative strains may be explained by the lipophilic characteristic of the quinone antibiotics. This property does not allow them to penetrate the outer membrane of Gram-negative strains in order to reach their intracellular targets. The inactivity shown by diospyrin, against the Gram-negative bacteria, could also be due to its lipophilic characteristics.

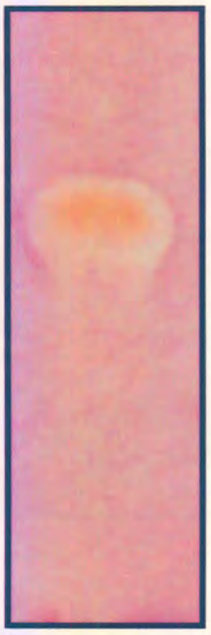

Figure 6.6 Zone of inhibition of *Staphylococcus aureus* on TLC plate in a direct bioassay of diospyrin isolated from the roots of *E. natalensis.* Silica gel 60 plate developed in chloroform-hexane(1:1).

A strong antibacterial activity was detected when plumbagin, a p-naphthoquinone with a phenolic group, isolated from the roots of *Plumbago auriculata* and *P. zeylanica* was tested against *Klebsiella aerogenes*, *S. aureus, B. aureus* and *B. cereus* (Van der Vijver & Lotter 1971) (Figure 6.7). In another study, it was found that diosquinone isolated from *Diospyros tricolor* showed significant activity at a low concentration (0.01 to 0.06 mg/ml) against Gram-positive bacteria (Alake 1994). The naphthoquinone, kigelinone, isolated from *Kigella pinnata* synonymous to *K. africana* have been shown to be active only against Gram-positive bacteria at concentrations ranging from 0.05 to 0.2 mg/ml.

The authors attributed this activity to the position of a hydroxyl group on the aromatic ring of the naphthoquinone nucleus. The position of the hydroxy group relative to the quinone carbonyl as in kigelinone makes the formation of chelation with an active site possible (Bintu *et al.* 1996) (Figure 6.8). Our findings reported here, would seem to support such a hypothesis, based on the fact that diospyrin also has a hydroxyl group adjacent to the quinone carbonyl.

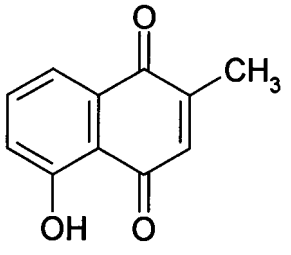

Figure 6.7 Plumbagin isolated from *Plumbago auriculata* and *P. zeylanica*

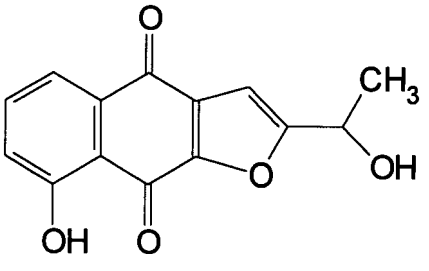

Figure 6.8 Kigelinone isolated from *Kigelia pinnata*

6.4 Conclusion

The traditional use of *E. natalensis* extract against sores, purulent lesions and skin infections could possibly be attributed to the activity of diospyrin against *S. aureus* and other pathogenic Gram-positive bacteria. This might explain the plant's use in traditional remedies against tuberculosis and adds credibility to earlier reports of the beneficial antimicrobial, antitrypanosomal and antiprotozoal effect of this binaphthoquinone (Khan *et al.* 1978; Yardley *et al.* 1996). The antimycobacterial properties of the root extract probably also provides the reason for its use in folk medicine to treat leprosy which is caused by another mycobacterial species (Watt & Breyer-Brandwijk 1962; Bryant 1966). The ability of diospyrin to inhibit the growth of all the Gram-positive bacteria tested as well as *M. tuberculosis*, shows the broad spectrum antibacterial activity of the compound.

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CHAPTER 7

ANTIVIRAL PROPERTIES OF *EUCLEA NATALENSIS*

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ANTIVIRAL PROPERTIES OF *EUCLEA NATALENSIS*

Abstract

The inhibitory effect of the crude extracts of *Euclea natalensis* A. DC. and a naphthoquinone, diospyrin isolated from this species was investigated against the herpes simplex virus type-1 (HSV-1) for activity by *in vitro* studies on primary vervet monkey kidney (VK) cells. Cell toxicity was monitored by determining the effect of the extracts and diospyrin on monolayers of VK cells. The concentrations of the plant extracts (ID_{50}) that inhibited 50% cell growth after the incubation period was found to be 0.1, 0.2 and 0.02 mg/ml for acetone, water extracts and diospyrin respectively. Water extract from the roots of the plant was the least toxic to cell cultures and inhibited the replication of HSV-1 moderately at a concentration of 0.2 mg/ml. Diospyrin had no inhibitory effect against the virus but the acetone extract inhibited the replication of the virus at concentrations ranging from 0.1 to 0.02 mg/ml as shown by the reduction of virus induced cytopathogenic effect and the protection of cells in the MTT assay (3-4, 5-dimethylthiazol-2,5-diphenyl tetrazolium bromide).

7.1 Introduction

Acute viral infections are world-wide in distribution because viruses have resisted prophylaxis or therapy longer than any other form of life. This is due to the nature of these infectious agents, which totally depend upon the cell they infect for their multiplication and survival. This unique characteristic has made it very difficult to develop effective chemotherapeutic agents for the treatment of viral infections. As a consequence, there are, at the moment only a few antiviral drugs available for the treatment of virus diseases (Garcia *et al.* 1999)

Acute and recurrent HSV-1 infections remain a serious problem both in developed and developing countries, due to their morbidity and mortality. HSV-1 produces skin infections and causes oral, facial cutaneous, esophageal and cerebral diseases which may result from a primary infection or, alternatively, from a reactivation of a latent infection. The infection is more serious in patients with deteriorated cellular immunity, who must receive chronic therapy with antivirals, favouring the selection of resistant variants (Kott *et al.* 1999). HSV-1 causes infections ranging from asymptomatic to life-threatening diseases even in the presence of humoral and cell-mediated immunity. HSV-1 infections may lead not only to symptomatic primary diseases but also to the development of recurrent lesions following reactivation of the latent virus in the neurons of the sensory ganglia.

The development of a new antiviral drug is a difficult task taking into account the poor selective toxicity and fast selection of resistant viral variants with the existing drugs. Frequencies of viral resistance to antiviral drugs are increasing and the difficulty of virus latency remains unsolved. The search for selective antiviral agents has been vigorous in recent years but the need for new antiviral therapies still exists since many of the problems relating to the treatment of HSV-1 infections still persists.

The emergence of drug resistant mutants of HSV-1 coupled with the toxicity of drugs towards uninfected cells and their prohibitive costs has been the reason for an extended search for new drugs. Less toxic and relatively cheap drugs, particularly from the traditional systems of medicine are needed (Padma *et al.* 1998). The screening of plants as a possible source of antivirals has led to the discovery of potent inhibitors of *in vitro* viral growth and the use of an ethnopharmacological approach enhances the probability of identifying new bioactive plant compounds (Send *et al.* 1996). Ethnopharmacological screenings of medicinal plants from China, Indonesia, Japan, Britain and many countries in Africa led to the selection of several extracts active towards herpes virus. For example, anthraquinones, alkaloids, lignans, flavonoids, phloroglucinol and catechin derivatives were found to have an inhibitory activity against the replication of cytomegalovirus or HSV viruses (Vanden Berghe *et al.* 1986; Tabba *et al.* 1989). These plant products are especially useful because of their abundance in nature and also sometimes for their low cytotoxicity.

Euclea natalensis is widely known in folk medicine for the relief of itches. Its extract is also used in a disinfectant bath apart from being used for chest diseases (Pujol 1990). The Shangaans use the charred and powdered root as an application to skin lesions in leprosy (Watt and Breyer-Brandwijk 1962; Bryant 1966). However, the validity of its medicinal properties against HSV-1 has not been evaluated scientifically. In this pilot study the antiviral effect of the crude extracts of *E. natalensis* and a naphthoquinone, diospyrin isolated from it was investigated for activity by *in vitro* studies on primary vervet monkey kidney (VK) cells.

7.2 Materials and methods

7.2.1 Plant material

Acetone and water extracts of *E. natalensis* were prepared as mentioned in chapter 2 (section 2.2.2). The acetonic and water residues were dissolved in DMSO and distilled water respectively to obtain a concentration of 500 mg/ml from which aliquots were diluted with Eagle's minimum essential medium (MEM) (Highveld Biological (Pty) Ltd., Kelvin, South Africa), to obtain the desired concentrations.

7.2.2 Cell culture and virus stock solution

Plant extracts were evaluated for toxicity against VK cells according to standard cell culture techniques, as outlined by Grist *et al.* (1979). Monolayers of VK cells were prepared by seeding 25 cm^3 flasks or 96-well microtitre trays with 10^5 cells/ml as mentioned in chapter 4 (section 4.2.2).

A stock suspension of virus, with a titre of $10^{6.5}$ $TCID_{50}$/ml, was prepared from a clinical isolate of HSV-1. The virus suspension was diluted in maintenance medium (MM) and used at a final concentration of 100 $TCID_{50}$/ml.

7.2.3 Cytotoxicity assay

Cell toxicity was monitored by determining the effect of the plant extracts on cell morphology and cell viability. Monolayers of VK cells were prepared separately in 96-well microtitre trays by seeding each with 200 µl 10^5 cells/ml in 10% MEM. Doublings dilutions of acetone and water extracts from a concentration of 2.0 mg/ml to 0.01 mg/ml were prepared in MM. Doubling dilutions of diospyrin was prepared from a concentration of 0.2 mg/ml to 0.001 mg/ml.

The extracts and diospyrin were tested for cytotoxicity by exposing the monolayers of the cell cultures to 200 µl dilutions of the compound in MM at 37^0C. Monolayers of cells exposed to MM, without the addition of extracts were used as controls. Control experiments showed that the final concentration of DMSO (0.2%) in the media had no cytopathic effect (CPE). The cells were monitored over a period of 7 days. The morphology of the cells was inspected daily and observed for microscopically detectable alteration, i.e. loss of monolayer, rounding, shrinking of cells, granulations, and vacuolisation in the cytoplasm. Results were expressed as the concentration that inhibits 50% cell growth after the incubation period (ID_{50}).

7.2.4 Antiviral assay

Acetone and water extracts were tested for antiviral activity at the final concentrations of 0.1-0.005 mg/ml and 0.2-0.01 mg/ml respectively. Diospyrin was tested at concentrations ranging from 0.02 mg/ml to 0.001 mg/ml. The final concentration of virus in the assay was 100 $TCID_{50}$/ml. 100 µl of the different dilutions of extract solution and 100 µl of the viral suspension, both diluted in MM, were added simultaneously to monolayers of each cell culture in microtitre wells. As positive controls, cells were infected with the same concentration of virus but without the addition of plant extracts, and as a negative or cell control, only MM was added to the cells. Cells were examined daily by light microscopy for the appearance of a cytopathic effect (CPE). After seven days the enzyme activity of viable cells in the microtitres was measured by the conversion of MTT (3-4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide, Sigma Chemical Co.) to a purple formazan precipitate as described previously (Mosmann 1983; Van Rensburg *et al.* 1994). The absence of CPE at a specific concentration of compound at the same time that the corresponding positive control showed CPE, was considered to be indicative of antiviral activity.

7.2.4.1 Preparation of virus staining solutions and the staining procedure

The viral staining procedure was done as follows:

- 100 mg of MTT powder was dissolved completely in 20 ml of phosphate buffer saline (PBS) powder. The solution was filtered and sterilised and kept in dark until used, as MTT is light sensitive.
- 20 µl of MTT mixture was added to each microtitre well.
- The microtitres were then incubated for 4 h at 37^0C.
- The microtitre plates were centrifuged at 720 x *g* for 10 min.
- Supernatant was carefully removed.
- 150 µl of PBS was added to each microtitre well.
- The plates were centrifuged again for the second time at 720 x *g* for 10 min and supernatant was removed.
- 200 µl of 0.2% DMSO were added to all the microtitre wells.
- The plates were shaken in the shaker for 1 h.
- The absorbance was read by a spectrophotometer at a wavelength of 540 nm using a reference wavelength of 620 nm.

7.3 Results and Discussion

The ID_{50} of the plant samples, the concentrations that inhibited 50% cell growth after the incubation period in the cytotoxicity assay was found to be 0.1, 0.2 and 0.02 mg/ml for acetone extract, water extract and diospyrin respectively (Table 7.1).

In the antiviral assay it was found that the acetone extract of *E. natalensis* showed moderate antiviral activity against HSV-1 at concentrations varying from 0.1-0.02 mg/ml. At these concentrations a cytopathic effect of ≤ 50%was observed on the cells in the MTT assay. Water extract exhibited weak activity at a concentration of 200 µg/ml, which corresponded to a 42% cytopathic effect. The inhibitory activity as shown in Table 7.1 exhibited a dose-dependent relationship.

Table 7.1 Dose response pattern of herpes simplex virus type-1 on VK cells to plant extracts and diospyrin. Absorbance of the negative cell controls for acetone extract, water extract and diospyrin was 0.518 ± 0.039, 0.503 ± 0.070 and 0.457 ± 0.063 respectively

Extracts and the isolated compound	Concentration (mg/ml)	[a]Absorbance	Cytopathic effect (%)
Acetone	0.1	0.370 ± 0.077	28.6
	0.05	0.311 ± 0.037	40.0
	0.02	0.291 ± 0.017	43.9
	0.01	0.255 ± 0.039	50.8
	0.005	0.220 ± 0.026	57.6
	+ve control	0.127 ± 0.017	75.5
Water	0.2	0.295 ± 0.031	42.0
	0.1	0.179 ± 0.034	64.5
	0.05	0.150 ±0.015	72.2
	0.02	0.112 ± 0.011	77.8
	0.01	0.133 ±0.018	73.6
	+ve control	0.124 ± 0.027	75.4
Diospyrin	0.02	0.123 ± 0.025	73.1
	0.01	0.098 ±0.011	78.6
	0.005	0.083 ± 0.023	82.0
	0.002	0.069 ± 0.014	85.0
	0.001	0.084 ± 0.008	82.0
	+ve control	0.104 ± 0.006	77.3

[a]Each value is the mean ± standard deviation of three readings.

Our results are consistent with the reports of other researchers. Antiherpes activity has been demonstrated for a number of plant extracts (Serkedjieva *et al.* 1990; Beucher *et al.* 1994; McCutcheon *et al.* 1995). *Polygonum punctatum, Lithraea molleoides, Sebastiana brasiliensis* and *Sebastiania klotzschiana* showed *in vitro* antiherptic activity with the 50% effective dose (ED_{50}) ranging from 39 to 169 µg/ml (Kott 1999). Biflavonoids, amentoflavone and robustaflavone isolated from *Rhus succedanea* exhibited moderate anti-HSV-1 activities with ED_{50} values of 17.9 and 11.6 µg/ml respectively (Lin *et al.* 1999). Complete inhibition of HSV-1 was observed when crude seed extract of *Pongamia pinnata* was evaluated for antiviral properties at a concentration of 1 mg/ml (Elanchezhiyan *et al.* 1993). The inhibitory effect of the flavonoids, iridoids, phenolic acids and saponins present in the flowers of *Verbascum thapsiforme* occurred at 190 µg/ml (Slagowska *et al.* 1987). The minimum inhibitory concentration of ethanolic extract of *Annona muricata* and water extract of *Petunia nyctaginiflora* was found to be 1 mg/ml (Padma *et al.* 1998). In the present study it was found that the majority of the antiviral activity was present in the acetone extracts. Traditionally, plant extracts are prepared with water (for example, infusions, decoctions and poultices), so it would seem unlikely that the traditional healer extracts those compounds which are responsible for activity in acetone extracts.

The effectiveness of plant extracts and the mechanisms of their inhibitory effect on viruses have been studied earlier by researchers. In our study the acetone extract of *E. natalensis* was found to exhibit activity at a concentration which was less than its ID_{50} value against the virus. Similar findings were reported by Hayashi *et al.* (1994) in, *in vitro* studies of the essential oils against this enveloped virus. In another study the antiviral activity of plant extracts has been attributed to a partial inactivation of viral replication which could occur due to the binding of glycoproteins of the viral envelop to the active compounds in the extracts. In this way the extract could interfere with the initial stages of virus replication and the release of newly formed virions. Thus the direct interaction with virus capsid protein could be at least one of the modes of the inhibitory effect (Serkedjieva & Ivancheva 1999). Similarly, according to Nozaki *et al.* (1989), the antiviral activity of Amaryllidaceae alkaloids on HSV-1 was thought to be due to the inhibition of multiplication and not to the direct inactivation of extracellular

viruses. However, the mechanism of the antiviral effect of the acetone extract of *E. natalensis* is yet unknown.

Diospyrin, a naphthoquinone isolated from the acetone extract of *E. natalensis* had no inhibitory activity against the virus. Send *et al.* (1996) who investigated other naphthoquinones also found no antiviral activity from them. No activity was also detected when two new naphthoquinones, rhinacanthin-C and rhinacanthin-D isolated from *Rhinacanthus nasutus* were tested against respiratory syncytial virus (RSV), influenza A virus (Flu-A) and herpes simplex virus at concentrations between 0.02 and 0.003 mg/ml (Send *et al.* 1996). However, Vanden Berghe *et al.* (1986) have shown that the naphthoquinone, juglone was active against HSV-1 at 62.5 µg/ml. The limited activity found in the acetone and water extracts in this study could be due to the presence of juglone and/or other compounds in the plant. 7-Methyljuglone is one of the constituents of the root of *E. natalensis* (chapter 8).

7.4 Conclusion

The present study demonstrated that the ID_{50} values of acetone and water extracts of *E. natalenis* and diospyrin isolated from it were 0.1, 0.2 and 0.02 mg/ml respectively against primary vervet monkey kidney cells. In the antiviral assay the acetone extract showed moderate antiviral activity against HSV-1, whereas water extract exhibited weak activity. Diospyrin had no inhibitory effect and therefore, it can be speculated that the limited activity found in the crude extracts of *E. natalensis* could be due to the presence of other biologically active constituents in the plant.

7.5 References

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CHAPTER 8

ISOLATION AND IDENTIFICATION OF 7-METHYLJUGLONE, THE SECOND ANTIBACTERIAL COMPOUND ISOLATED FROM *EUCLEA NATALENSIS*

Chapter 8

ISOLATION AND IDENTIFICATION OF 7-METHYLJUGLONE, THE SECOND ANTIBACTERIAL COMPOUND ISOLATED FROM *EUCLEA NATALENSIS*

Abstract

A naphthoquinone (NQ) 7-methyljuglone, was isolated from *Euclea natalensis* A.DC. and evaluated for its activity against a drug-sensitive and drug-resistant strains of *Mycobacterium tuberculosis* and other bacterial species. The minimal inhibitory concentration (MIC), of 7-methyljuglone was found to be 50 µg/ml for both the strains of *M. tuberculosis*. The compound was also tested against five Gram-positive and six Gram-negative bacteria. The MIC was 50 µg/ml for *Bacillus pumilus, B. subtilis, Micrococcus kristinae* and 100 µg/ml for *B. cereus* and *Staphylococcus aureus*. No inhibitory effect of the compound was observed on any Gram-negative bacteria at the highest concentration (100 µg/ml) tested. The synergistic effect of two naphthoquinones (NQs), diospyrin and 7-methyljuglone, was evaluated on *M. tuberculosis* and other bacterial species. A significant synergistic effect of the combination of the two NQs was observed. The MIC of the combined treatment was 10 µg/ml on both strains of *M. tuberculosis*. Marked improvement in efficacy of the NQs was also observed when the compounds were tested in combination against the other bacteria. The MIC was 50 µg/ml for all Gram-positive bacteria investigated. No synergistic effect was observed on any Gram-negative bacterial species investigated.

8.1 Introduction

The presence of biologically active naphthoquinones (NQs) in plants, fungi, lichens, and echinoderms (mainly in sea urchins) has been known for a long time. Antibacterial and antifungal activity of NQs, isolated from various plants have been reported previously (Croft *et al.* 1992; Khan & Timi 1999). In addition a number of NQs have been found to be haemostatic (Khan *et al.* 1978).

The discovery of the tubercle bacillus by Robert Koch in 1882 intensified the search for an effective and specific drug against TB. In the presently available drug-regimes which comprises of 4-5 drugs administered in combination for 6-9 months duration, the patients frequently stop taking drugs as soon as the symptoms are ameliorated and the treatment is therefore discontinued. A specific drug is needed which can cure TB in a much shorter time. Today, many bactericidal and bacteriostatic drugs are used in combination all over the world including in Western medicine and traditional medicine, with dramatic positive results. The rise of multidrug-resistant TB (MDR-TB) has become a serious problem as it is very difficult to treat and requires a longer period of treatment (National Jewish Medical and Research Centre 1994). These facts are prompting researchers to search for a potentially active combination of drugs against these strains which can cure TB in a much shorter time.

The Ebenaceae family is in particular well known as a source of NQs but not much is known about their antimycobacterial activity. During our investigation of South African medicinal plants, species used traditionally in the treatment of TB was identified and recognized. A significant activity of the crude extracts of *Euclea natalensis* A. DC. (Ebenaceae) and a naphthoquinone (NQ), diospyrin, isolated from this plant was observed against drug-sensitive and drug-resistant strains of *M. tuberculosis* and against other bacterial species (Lall & Meyer 1999; Meyer

& Lall 2000). It was decided to investigate *E. natalensis* for the occurrence of another active compound and determine if it has activity against *M. tuberculosis.* Activity guided fractionation of the acetone extract of *E. natalensis* indicated earlier that there could be another compound present in the plant with antimycobacterial activity.

Over the last 25 years, an intensive effort has been made to uncover, clinically useful, antibiotics. Certain diseases, however, remain serious problems and some of the major antibiotics have considerable drawbacks in terms of a limited antimicrobial spectrum or serious side effects. These factors impel a continuing search for new antibiotics that are safe and effective against clinical infections caused by Gram-positive and Gram-negative organisms, fungi as well as viruses (Mitscher *et al.* 1972). However, in recent developments, new products or synergistic combinations of drugs have resulted with positive results (Croft *et al.* 1992).

The aim of this study was to isolate another compound from *E. natalensis* and determine its minimal inhibitory concentration (MIC) for *M. tuberculosis* and other bacterial species. It was also decided to evaluate the synergistic effect of the two NQs, diospyrin and 7-methyljuglone on five Gram-positive and six Gram-negative bacterial species and on drug-sensitive and drug- resistant strains of *M. tuberculosis.*

8.2 Materials and Methods

8.2.1 Extraction, isolation and purification of the compound

Acetone extract of the roots of *E. natalensis* was prepared as mentioned in chapter 6 (section 6.2.1). Through the activity-guided fractionation of the acetone extract of *E. natalensis,* attempts were made to isolate a compound from the fraction possessing antimycobacterial activity. In order to isolate the compound the crude extract of the plant (4.2 g) was dissolved in $CHCl_3$ and fractionated on silica gel 60 by column chromatography, using $CHCl_3$ as eluent. The bioactive antimycobacterial fraction was

then subjected to a silica gel 60 column in a biotage flash chromatography apparatus, using chloroform:hexane (1:1) as eluent (Figure 8.1). The compound was finally purified on silica gel 60 column, using hexane: ethyl acetate (1:1) as eluent (Figure 8.2).

^{1}H-NMR, ^{13}C-NMR, and DEPT spectra were obtained at 400/100 MHz on an AMX 400 NMR spectrometer using $CDCl_3$, as solvent with TMS as internal standard. The mass spectra was obtained with a JEOL JMS-AX505 W High resolution double focussing mass spectrometer. Data was processed using the Complement data system supplied by JEOL on an HP500 computer.

8.2.2 Bioassay on *M. tuberculosis*

The radiometric respiratory technique with the BACTEC apparatus was used for susceptibility testing of *M. tuberculosis.* Bacterial cultures utilized in this study were grown from specimens received from the Medical Research Council (MRC) in Pretoria. A sensitive strain of *M. tuberculosis*, H37Rv reference strain and a strain (MRC strain no. CCKO28469V) resistant to two drugs, isoniazid and rifampin were used in the screening procedure (Table 8.1).

7-Methyljuglone was dissolved at 10 mg/ml in 1% DMSO and stored at -4^{0}C until used. Subsequent dilutions were done in DMSO and added to 4ml of BACTEC 12B broth to achieve the desired final concentrations of 100, 50 and 10 μg/ml together with PANTA (Becton Dickinson & Company), an antimicrobial supplement. The BACTEC drug susceptibility testing was also done for the two primary drugs streptomycin and ethambutol at concentrations of 6 μg/ml and 7.5 μg/ml respectively, against the H37Rv sensitive strain. Preparation of bacterial cultures and the testing procedures were the same as described in chapter 3 (section 3.2.2). All tests were done in triplicate.

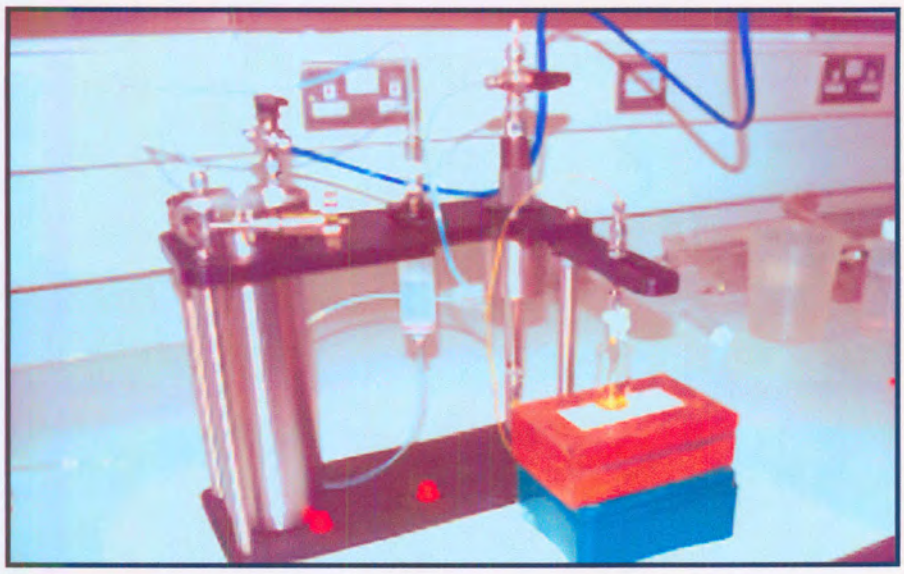


Figure 8.1 Biotage flash chromatography apparatus

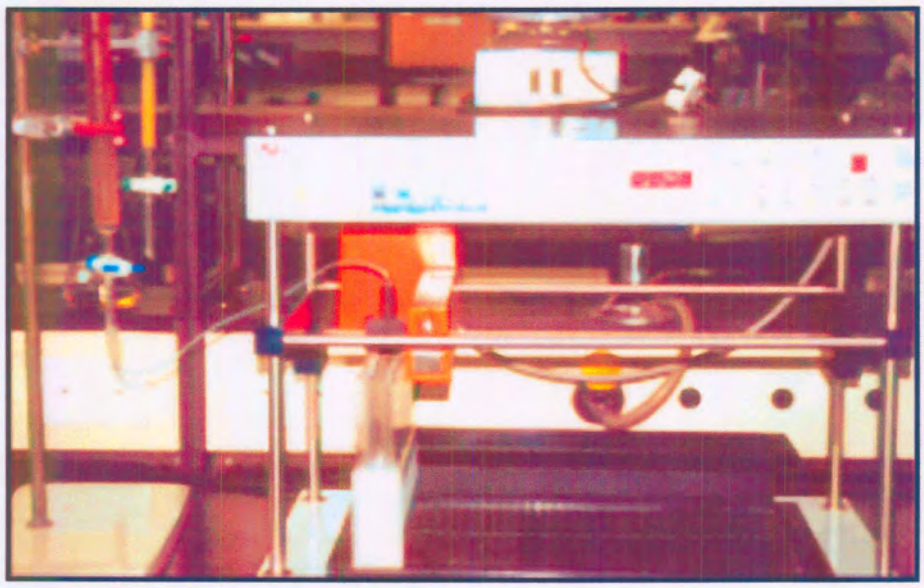


Figure 8.2 Glass column chromatography and fraction collector

The synergistic effect of the two naphthoquinones diospyrin, and 7-methyljuglone was also determined at final concentrations of 100.0, 50.0, 10.0 and 5.0 μg/ml of each compound against the H37Rv sensitive strain of *M. tuberculosis* and a strain resistant to the two drugs, isoniazid and rifampin.

8.2.3 Bioassay on other Bacterial species

7-Methyljuglone was tested for inhibitory activity against five Gram-positive and six Gram-negative bacterial strains. Gram-positive bacterial species analysed were *Bacillus cereus, B. pumilus, B. subtilis, Micrococcus kristinae, Staphylococcus aureus*, and the Gram–negative bacterial species *were Enterobacter cloaccae, E. aerogenes, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Serratia marcescens*. Bacterial species were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria.

The MIC of 7-methyljuglone was determined by incorporating various amounts (100.0, 50.0, 1.0 and 0.5 μg/ml) of the solution containing the compound, into petri dishes containing the culture media as done previously by Meyer and Dilika (1996) and described in chapter 5 (section 5.2.4). Two blank plates containing only nutrient agar and two containing nutrient agar and 1% acetone without the compound, served as controls to ensure the validity of the test. In addition two plates containing streptomycin sulfate at concentrations of 100.0, 50.0 and 10.0 μg/ml served as positive controls. The MIC was regarded as the lowest concentration of the extracts that did not permit any visible growth when compared with that of the controls.

The synergistic effect of the two NQs, diospyrin and 7-methyljuglone was also determined at final concentrations of 100.0, 50.0, 10.0, 5.0 μg/ml each against the bacterial species.

8.3 Results and Discussion

8.3.1 Identification of the isolated compound

The compound was identified as the naphthoquinone, 7-methyljuglone, by comparison of its ^{1}H-NMR and ^{13}C-NMR spectral data with published spectra (Tezuka *et al.* 1973; Sankaram *et al.* 1986) (Figure 8.3). 7-methyljuglone is a monomer of diospyrin previously isolated from *E. natalensis*.

The ^{1}H-NMR spectrum exhibited signals at L 2.43 from one methyl group, 11.86 from the hydroxyl group, one proton singlets at 7.08, 7.44 and a doublet at 6.91 (Figure 8.4). The ^{13}C NMR spectrum of 7-methyljuglone shows eleven signals corresponding to its molecular formula. The chemical shifts are assigned in analogy with juglone (Sankaram *et al.* 1986). The signals of the unsubstituted carbons C-2, C-3, C-6 and C-8 are more intense and can be distinguished from the weak lines C-1 (>C=O), C-4 (>C=O), C-5, C-7, C-9 and C-10. The methyl carbon is also moderately intense (Figure 8.5). DEPT spectra exhibited the presence of four CH and one CH_3 group in the compound (Figure 8.6). The mass spectra of 7-methyljuglone can be correlated with those of simple 1,4 NQs but show a more complex pattern of mainly low-intensity peaks (Figure 8.7). The molecular weight M+=188.0455, determined by mass spectroscopy, corresponds to a molecular formula of $C_{11}H_8O_3$. The percentage composition of the elements carbon, hydrogen and oxygen in the pure crystals and the mass of the molecular ion are in accordance with the molecular formula. In the mass spectra, the initial loss of a methyl radical is dominant followed by successive loss of CO.

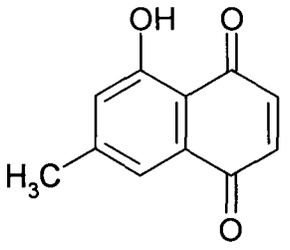


Figure 8.3 Structure of 7-methyljuglone

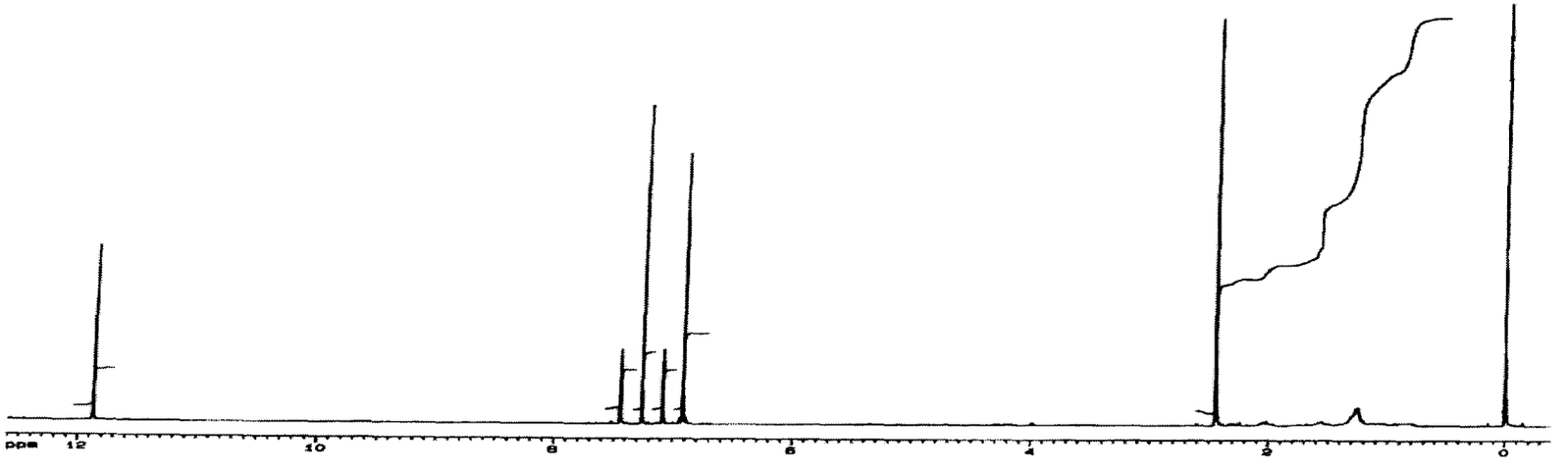


Figure 8.4 ^{1}H-NMR spectrum of 7-methyljuglone isolated from *E. natalensis*

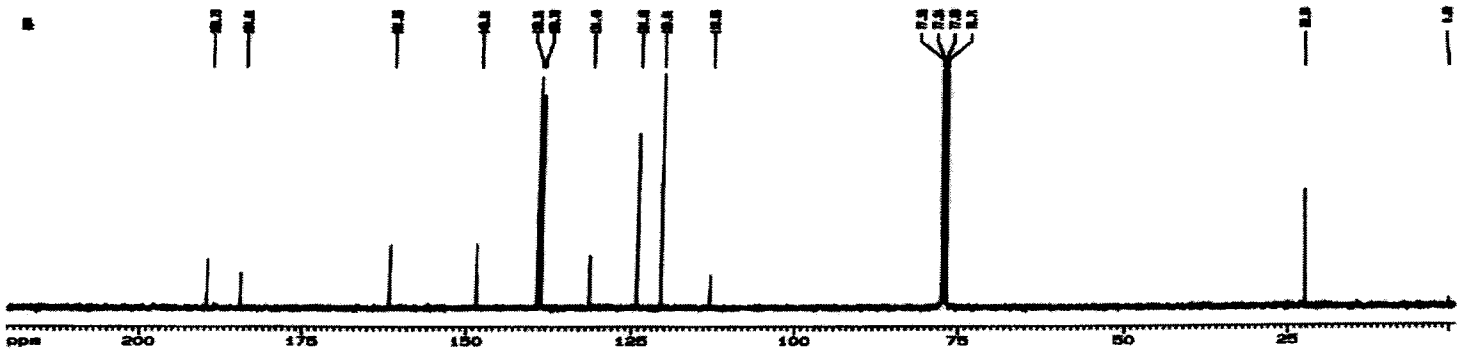


Figure 8.5 ^{13}C-NMR spectrum of 7-methyljuglone isolated from *E. natalensis*

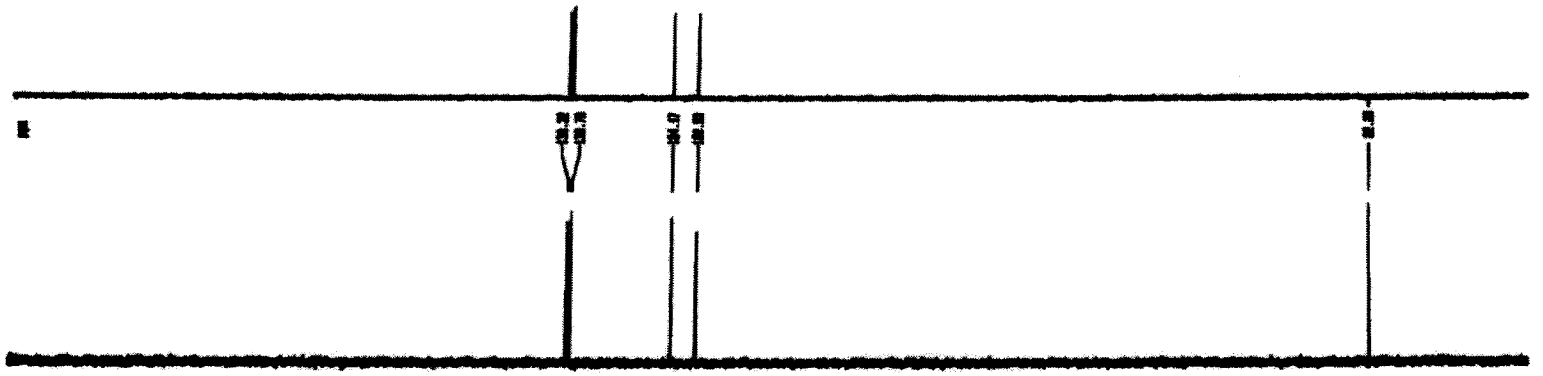


Figure 8.6 DEPT spectra of 7-methyljuglone isolated from *E. natalensis*

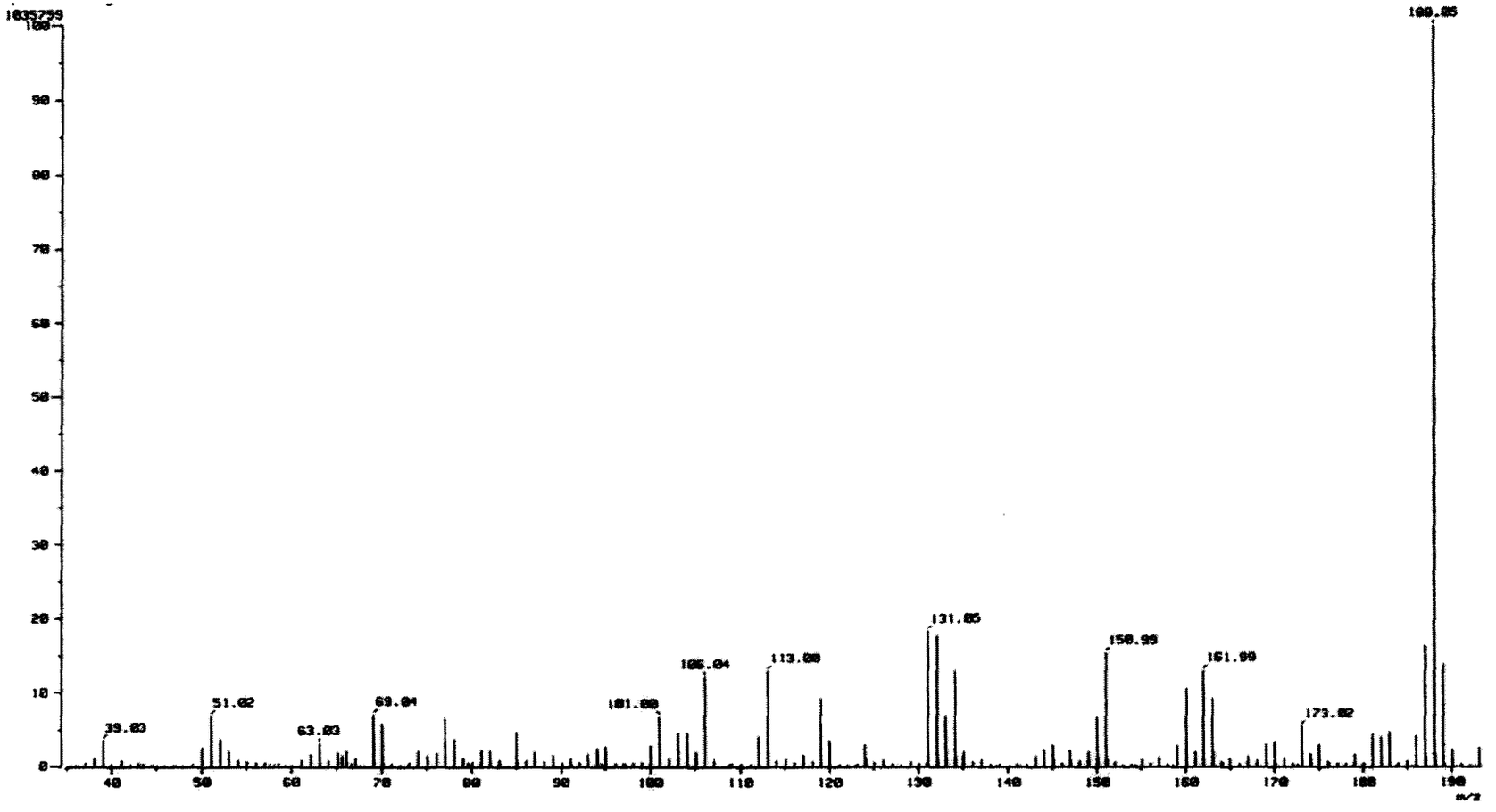


Figure 8.7 Mass spectra of 7-methyljuglone isolated from *E. natalensis*

7-methyljuglone has also been isolated previously from *E. natalensis* (Van der vijver & Gerritsma 1976) and several other species from Ebenaceae family such as *E. pseudebenus* (Ferreira *et al.* 1973), *E. crispa, E. undulata* (Van der vijver & Gerritsma 1976) and *E. divinorum* (Costa *et al.* 1976), and from the *Diospyros* species, *D. elliptifolia* (Fallas & Thomson 1968), *D. ferrea* var. *buxifolia, D. japonica, D. kaki, D. lotus* (Tezuka *et al.* 1973) and *D. chamaethamus* (Costa *et al.* 1998). These plants are commonly used in traditional medicine for various pathological conditions.

8.3.2 Effect of 7-methyljuglone as a single agent and in combination with diospyrin on *M. tuberculosis*

Results were interpreted on day 6 or 7 when the control vials containing the 1:100 dilution of the inoculum reached a GI value of 30 or more (Table 8.1). The MIC of 7-methyljuglone was found to be 50 µg/ml against the H37Rv strain as well as against the drug-resistant strain. However, the ΔGI values of the vials containing 7-methyljuglone at 10 µg/ml concentration level, for the two drug-resistant strain was found to be 20.33 ± 7.63. This indicated that this strain is partially susceptible to the compound at a low concentration of 10 µg/ml. The ΔGI values of the vials containing streptomycin (6 µg/ml) and ethambutol (7.5 µg/ml) were found to be 3 ± 2.12 and 6 ± 4.41 respectively. Marked efficacy of the NQs, diospyrin and 7-methyljuglone was observed in synergy. The MIC was found to be 10 µg/ml against the H37Rv and the drug resistant strain.

Various compounds with antimycobacterial activity, such as alkaloids, flavonoids, oils, triterpenes, sesquiterpenes, monoterpenoids and naphthoquinones isolated from medicinal plants have been reported earlier (Cantrell 1996; Ulubelen *et al.* 1997; Houghton 1999; Lall & Meyer 1999). The MIC of the acetone extract of *E. natalensis* was found to be 0.1 mg/ml against a drug-sensitive and a two drug-resistant strains of *M. tuberculosis* (Lall & Meyer 1999). In our study it was found that the monomer of diospyrin, 7-methyljuglone had more activity against the bacteria than diospyrin and crude extract of *E. natalensis*. It is interesting to note that the MIC of 7-methyljuglone was 50 µg/ml against both the strains of *M. tuberculosis* whereas

Table 8.1 Effect of 7-methyljuglone as a single agent and in combination with diospyrin on the growth of the sensitive strain (H37Rv) and resistant strains of *Mycobacterium tuberculosis* as determined by the radiometric method

Mycobacterium tuberculosis strains	Lab reference no.	Compound(s) (treatment)	MIC[a] (µg/ml)	ΔGI[b] values of plant extracts	ΔGI values of the control vial
H37Rv sensitive strain	ATCC27294	7-methyljuglone	50	0 ± 1	15 ± 3.78
Two drug (isoniazid and rifampin) resistant strain	CCKO28469V	7-methyljuglone	50	0 ± 0	30 ± 4.94
H37Rv sensitive strain	ATCC27294	Diospyrin+7-methyljuglone	10	3 ± 1	15 ± 3.78
Two drug (isoniazid and rifampin) resistant strain	CCKO28469V	Diospyrin +7-methyljuglone	10	3.33 ± 3.05	30 ± 4.94

[a]minimal inhibitory concentration.

[b]ΔGI values are means ± standard deviation.

diospyrin inhibited the growth of the organisms at 100 μg/ml. Another naphthoquinone plumbagin which is closely related to 7-methyljuglone, was found to have more activity against *Leishmania* and *Typanosoma cruzi* as compared to the dimer of plumbagin (Boza & Cassels 1995).

There are very few reports of medicinal plants being investigated against the MDR-TB strains. Antimycobacterial activity of 7-methyljuglone just as a single agent as well as in combination with diospyrin showed better activity against the MDR-TB strain than the other compounds previously reported. Essential oils present in *Eucalyptus botryoides* and *E. camadulensis* showed very weak activity against a MDR-TB strain even at a high concentration of 10 mg/ml and 5 mg/ml respectively. Oils of other *Eucalyptus* species, such as *E. maculata, E. tereticornis* and *E. leptocephala* also showed similar activity (Morerira *et al.* 1997; Leite *et al.* 1998).

It has been suggested that most isolates of *M. tuberculosis* as well as *M. avium* are resistant to first-line anti-tuberculous drugs when tested individually but they are susceptible *in vitro* to certain combinations of these drugs. A combination of diospyrin and 7-methyljuglone showed marked synergy in our study. Several studies indicate therapeutic success of certain drug combinations. Clinical trials of a hydroxynaphthoquinone with synergistic drug combinations proved to be effective on prophylaxis of *Pneumocystis carinii pneumonia* in a mouse model (Comley & Sterling 1995). In another study the combination of two NQs, atovaquone and proguanil has been found to be more effective in treating malaria than when either of the two was used (Srivastava & Vaidya 1999). The combination of the plant natural products, curcumin and genistein, have the potential to reduce the proliferation of estrogen-positive cells and thereby preventing hormone related cancers in humans. The synergistic inhibitory effect of these compounds resulted in total inhibition of cell growth (Verma *et al.* 1997).

A pure agent which is active *in vitro* at a concentration ≤ 0.1 mg/ml is likely to be a candidate for clinical use (Mitscher *et al.* 1972). It can be expected that an integrated approach in using a combination of NQs to combat MDR-TB strains, might

therefore, uncover important lead compounds for the development of improved drugs which are sorely needed to control TB.

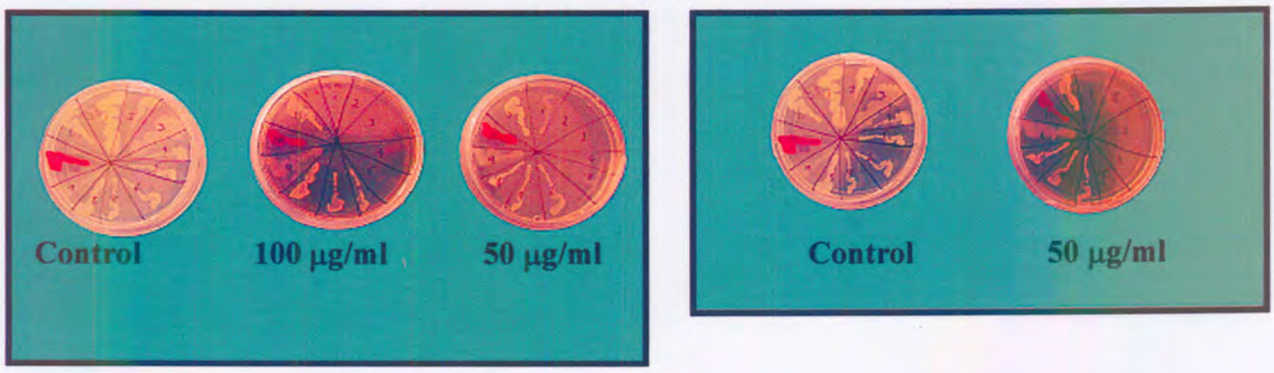
8.3.3 Effect of 7-methyljuglone as a single agent and in combination with diospyrin on other bacterial species

The antibacterial investigation of 7-methyljuglone showed that the growth of the Gram-positive organisms, *Bacillus cereus, B. pumilus, B. subtilis, Micrococcus kristinae* and *Staphylococcus aureus* was inhibited at a concentration varying from 50-100 µg/ml (Figure 8.8). No inhibitory effect of the compound was observed on any Gram-negative bacteria at the highest concentration (100 µg/ml) tested. Marked improvement in efficacy of the NQs was also observed when the compounds were tested in combination against the Gram-positive bacteria. No synergistic effect was observed on any Gram-negative bacterial species investigated (Table 8.2) (Figure 8.8). The reference antibiotic, streptomycin sulfate inhibited the growth of all the bacterial species tested in this study at 10 µg/ml except, *Pseudomonas aeruginosa* and *Serratia marcescens* which were inhibited at 50 µg/ml and 100 µg/ml respectively.

Table 8.2 Antibacterial activity of 7-methyljuglone as a single agent and in combination with diospyrin, isolated from the roots of *E. natalensis*

Bacterial species	Gram +/-	MIC[a] of 7-methyljuglone (µg/ml)	MIC of 7-methyljuglone+diospyrin (µg/ml)
Bacillus cereus	+	100	50
B. pumilus	+	50	50
B. subtilis	+	50	50
Micrococcus kristinae	+	50	50
Staphylococcus aureus	+	100	50
Enterobacter cloacae	-	na[b]	na
E. aerogenes	-	na	na
Escherichia coli	-	na	na
Klebsiella pneumoniae	-	na	na
Pseudomonas aeruginosa	-	na	na
Serratia marcescens	-	na	na

[a]minimal inhibitory concentration.
[b]not active.



(a) (b)

Figure 8.8 Effect of 7-methyljuglone as a single agent and in combination with diospyrin, on bacterial species: (a) 7-methyljuglone (b) 7-methyljuglone + diospyrin.

NQs in general are well known for antibacterial, antifungal and antitumoural activities. From a dichloromethane extract *of Newbouldia laevis* roots four new and six known NQs were isolated and all NQs showed antifungal activity. The concentrations required to inhibit the growth of the bacteria *B. subtilis* and *E. coli* varied from 1.25 µg/ml to 40 µg/ml (Gafner *et al.* 1996). It was found that a new quinone, gunacin exhibited good inhibitory effect against *S. aureus, Streptococcus aronson* and *S. pyrogenes* at concentrations varying between 0.02 µg/ml to 0.09 µg/ml (Werner *et al.* 1979). The diosquinone isolated from *Diospyros mespiliformis* has been shown to have antibacterial, antimalarial and antiprotozoal activity (Lajubutu *et al.* 1995; Yardley *et al.* 1996). The antibacterial properties of *Lawsonia inermis* have been attributed to the presence of naphthoquinones (Wren 1998).

In previous studies 7-methyljuglone have been reported for its inhibitory effect on various pathogenic organisms such as *Klebsiella aerogenes, Shigella dysenteriae, S. flexnerii, Cornebacterium diphtheriae, Neisseria gonorrhoeae* etc. During an investigation of the antibiotic action of root bark of *E. natalensis* zones of inhibition for *B. cereus* and *S. aureus* were observed to be 9 mm and 11 mm respectively at a concentration of about 0.3 mg/ml (Khan *et al.* 1978). In the present study total inhibition of these two bacteria together with other bacterial species *B. pumilus, B. subtilis* and *M. kristinae* was found even at a lower concentration of 0.1 mg/ml. Another very similar NQ, plumbagin, an isomer of 7-methyljuglone and bisplumbagin, has shown antileishmanial activity. The antiprotozoal activities of hydroxynaphthoquinoids attracted attention when some synthetic compounds of this class were identified as potential drugs against several protozoal parasitic diseases (Croft *et al.* 1992). During the investigation of antibacterial activity by disc diffusion method it was found that when plumbagin (1mg/disc) was tested, the zones of inhibition of *B. cereus, B. subtilis, E. coli, M. luteus, Salmonella typhymurium* and *S. aureus* were between 8 and 18 mm (Khan & Timi 1999).

The possible effectiveness of *E. natalensis* against sores, purulent lesions and skin infections could be attributed to the activity of the naphthoquinones diospyrin and 7-methyljuglone against *S. aureus* and other Gram-positive bacteria. The above *in vitro* observations on the antibacterial activity of these naphthoquinones against the tested organisms justify further investigation on their curative properties. The antibacterial activity of 7-methyljuglone was found mainly against the Gram-positive bacteria. Synergistic combination of two NQs also did not show any activity against the Gram-negative bacteria. The negative results obtained against the Gram-negative bacteria were not surprising as, in general, these bacteria are more resistant than the Gram-positive ones (Martin 1995; Paz *et al.* 1995; Vlietinck *et al.* 1995). The outer membrane, a layer of the cell wall of Gram-negative bacteria is composed of lipopolysaccharide, phospholipids and proteins. This outer membrane apparently acts as a penetration barrier for various substances (Nikaido 1976). In classifying the activity of the antibiotic extracts as Gram-positive or Gram-negative, it would generally be expected that a much greater number would be active against Gram-positive organisms

than against Gram-negative. Our results are in correlation with the previous reports. In an antibiotic screening of medicinal plants of the British Columbian native peoples, a large number of extracts (70) were active against both Gram-positive and Gram-negative bacteria while only a relatively low number (9) were active against Gram-negative bacteria only (McCutcheon *et al.* 1992).

The mutagenecities of naturally occurring quinones such as plumbagin, juglone and 2-hydroxy-naphthoquinone were tested on *Salmonella typhimurium* strains and it was found that they were mutagenic to the organism with metabolic activation. Results also suggested that NQs are mutagenic when they have only one or two hydroxyl and/or methyl substituents (Tikkanen *et al.* 1983). According to Osman *et al.* (1983) the high lipid solubility of NQs facilitate the diffusion of the compounds through the cell wall giving them a better opportunity to exert their effects on the organism.

It is widely accepted that the action of many quinone compounds in cells is partially or completely due to their capacity to stimulate superoxide production which can be converted into hydroxyl radicals by transition metals such as Fe^{2+} and Cu^{2+}. This subsequently leads to DNA damage, thiol oxidation and lipid peroxidation (Afanas'ev *et al.* 1990). According to Haraguchi *et al.* (1997) the generation of superoxide anion and hydrogen peroxide in bacterial cells were due to the stimulation of NQs. NQs were found to substantially increase the potentially toxic H_2O_2 production in *Trypanosoma brucei.* The initiation of sublytic concentrations of both the naphthoquinone and a free radical initiator, heme led to a synergistic lysis of the organism *in vitro*. Bioactive hydroxy-naphthhoquinoids have been linked to co-enzyme Q (ubiquinone) and their activities attributed to the generation of specific free radicals through semiquinone intermediates, leading to lipid peroxidation (Meshnick *et al.* 1978). In this regard, evaluation of the increase of oxidative stress by unchecked production of superoxide anion radicals in *M. tuberculosis* by the two NQs, diospyrin and 7-methyljuglone might be useful. It might also be helpful to investigate if there is any change of antioxidant enzyme level in *M. tuberculosis* when determining the mechanism of anti-TB effect of the NQs.

8.4 Conclusion

This study has shown that the two active NQs of *E. natalensis* have strong *in vitro* antibacterial activity. The synergistic combination of NQs may be efficacious and could play an important role in the therapy of MDR-TB or other bacterial infections. Such combinations might improve naphthoquinones' efficacy and broaden its spectrum of activity. The reported antimycobacterial and antibacterial activity of *E. natalensis* can be attributed to the presence of 7-methyljuglone and diospyrin. Detailed knowledge of the interaction of antimycobacterial drugs may help substantiate the choice of certain drug combinations with these natural products and thus reduce the number of drugs needed for effective treatment.

8.5 References

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CHAPTER 9

GENERAL DISCUSSION AND CONCLUSION

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9.1 Motivation for this study

A renewed interest in evaluating the activity of a number of medicinal plants used in folk medicine against *Mycobacterium tuberculosis* has developed recently because of the recent upsurge in the incidence of TB with a significant emergence of multidrug-resistant (MDR) cases (Girling 1989; Grange & Davey 1990). People have become increasingly aware that about three quarters of the world's population continue to use and believe in the effectiveness of their traditional, mainly plant, remedies (Phillipson & Anderson 1989). The challenge of the ethnopharmacological approach to new drug development will be of future benefit to Western Medicine.

The detailed analysis of the pharmacological properties of medicines used traditionally in South Africa brought to light that the active ingredients present in plants do often have a specific pharmacological reaction towards sickness which are recognized by pharmacologists (Watt & Breyer-Brandwijk 1962; Hutchings 1966; Van Wyk *et al.* 1997). The use of medicinal plants by South Africans in curing TB related symptoms such as fever, cough, chest disease, night sweats etc. has been reported (Watt & Breyer-Brandwijk 1962; Pujol 1990; Hutchings 1996). Scientific basis for the utilisation of such plants in South African folk medicine was required and therefore, antimycobacterium activity of 20 local medicinal plants was investigated. Plant extracts were screened for activity against a drug-sensitive and a few drug-resistant strains of *M. tuberculosis*.

9.2 *In vitro* susceptibility testing of *Mycobacterium tuberculosis*

The techniques available for the scientific evaluation of biologically active compounds are becoming rapidly more sensitive and many *in vitro* test procedures are available for the primary screening of extracts. The antimycobacterial activity of South African medicinal plants were investigated using two methods commonly used these days; the conventional agar plate method and the BACTEC radiometric method for susceptibility testing of *M. tuberculosis*. The advantages and disadvantages of these two techniques were also compared and discussed.

In a preliminary testing 20 South African medicinal plants used to treat pulmonary diseases were screened for activity against *M. tuberculosis* by the conventional agar plate method. Acetone and water extracts of the plants were investigated for their inhibitory action against H37Rv, a drug-sensitive strain of *M. tuberculosis.* Fourteen of the 20 acetone extracts showed inhibitory activity at a concentration of 0.5 mg/ml. Six water extracts showed activity at concentrations ranging from 0.5 to 5.0 mg/ml.

Fourteen South African medicinal plants that showed good activity against *M. tuberculosis*, using the conventional agar method were screened again to confirm their inhibitory activity employing the BACTEC radiometric method. Acetone plant extracts were screened against the H37Rv strain as well as a strain resistant to the drugs isoniazid and rifampin. The minimal inhibitory concentration (MIC) of *Croton pseudopulchellus, Ekebergia capensis, Euclea natalensis, Nidorella anomala,* and *Polygala myrtifolia* was found to be 0.1 mg/ml against the H37Rv strain. Extracts of *Chenopodium ambrosioides, E. capensis, E. natalensis, Helichrysum melanacme, N. anomala* and *P. myrtifolia* were active against the resistant strain at 0.1 mg/ml. Eight plants showed activity against both the strains at a concentrations of 1.0 mg/ml.

A good correlation between the susceptibilty test results of the radiometric assay and conventional methods was observed. Susceptibility testing of *M. tuberculosis* by agar plate method is reliable, economical and reproducible. The advantage of BACTEC radiometric assay over the conventional method is that it is rapid and because of the liquid medium being used there is more cell to drug contact and hence, more accuracy in the results that that of the agar plate method.

9.3 Cytotoxicity assay of plant extracts

Cytotoxicity evaluation of the plant extracts and their active principles is required for its effective therapeutic use. After establishing the antimycobacterial activity of 14 plant extracts against *M. tuberculosis*, the next step was to isolate the active compound (s) from one of the most active and the least toxic plants. Fourteen South African medicinal plants were evaluated for their cytotoxic properties against primary vervet monkey kidney cells (VK). *Acacia xanthophloea, Chenopodium ambrosiodes* and *Ekebergia capensis* showed significant toxicity against VK cells exhibiting an ID_{50} between 0.7 to 6 µg/ml whereas the remaining plant extracts exhibited moderate cytotoxicity. The crude acetone extracts of *Euclea natalensis* was found to be the least cytotoxic. At a concentration of 0.1 mg/ml the VK cells did not exhibit altered morphology or growth characteristics indicative of a cytotoxic effect. *E. natalensis* was selected for the isolation of its active compound(s) because of its antimycobacterial properties and low cytotoxicity.

9.4 Antibacterial activity of the crude extract of *E. natalensis*

Roots of *E. natalensis* is widely used by indigenous people of South Africa for various pathological bacterial infections apart from being used in curing TB-symptoms. Powdered root bark is used as an ingredient in medicines to treat urinary tract infections, venereal diseases, glandular swellings, various chest diseases such as bronchitis, pleurisy, chronic asthma etc. (Bryant 1966; Pujol 1990).

It was therefore, decided to investigate the antibacterial activity of acetone and water extracts of *E. natalensis* against five Gram-positive and six Gram-negative

bacterial species. The water and acetone extracts inhibited the growth of *Bacillus cereus, B. pumilus, B. subtilis, Micrococcus kristinae* and *Staphylococcus aureus* at concentrations ranging between 0.1 and 6.0 mg/ml. The water extract did not exert any inhibitory action on Gram-negative bacteria while the acetone extract showed inhibitory activity at a concentration of 5.0 mg/ml against all the Gram-negative bacteria investigated.

9.5 Antimycobacterial and antibacterial activity of the isolated compounds from *E. natalensis*

Out of 14 plants tested for toxicity and activity it was found that *E. natalensis* possesses high antimycobacterial activity and is comparatively less toxic than the other plants investigated. Through the activity-guided fractionation of the acetone extract of *E. natalensis* two closely related bioactive compounds (diospyrin and 7-methyljuglone) were isolated and identified. MICs of the isolated compounds were determined for drug-sensitive as well as drug-resistant strains of *M. tuberculosis.* Synergistic inhibitory action of the compounds was also investigated against H37Rv, a drug-sensitive and a drug-resistant strain, resistant to two drugs isoniazid and rifampin of *M. tuberculosis*. The MICs of the isolated compounds for eleven bacterial species were determined separately for the individual compounds and the synergistic effect of the two compounds was also evaluated against the bacterial species.

The MICs of diospyrin and 7-methyljuglone were found to be 100 and 50 μg/ml respectively for all the *M. tuberculosis* strains. Diospyrin inhibited the growth of the Gram-positive organisms, *B. cereus, B. pumilus, B. subtilis, M. kristinae* and *S. aureus* at a concentration of 100 μg/ml. No inhibitory effect of the compound was observed on any Gram-negative bacteria at the highest concentration (100 μg/ml) tested. 7-methyljuglone inhibited the growth of the Gram-positive organisms, *B. cereus* and *S. aureus* at a concentration of 100 μg/ml whereas MICs for *B. pumilus, B. subtilis, M. kristinae* were found to be 50 μg/ml. No inhibitory effect of the compound was observed on any Gram-negative bacteria at the highest concentration (100 μg/ml) tested.

A significant synergistic effect of the two NQs was observed against the organisms. MICs obtained were 10 µg/ml and 50 µg/ml for *M. tuberculosis* and the bacterial species respectively. No synergistic effect was observed on any Gram-negative bacterial species investigated.

9.6 Antiviral activity of *E. natalensis*

It has been reported that extracts of the root of *E. natalensis* are believed to be used as a natural remedy for various skin infections by South Africans. The charred and powdered root is used as an application to the skin lesions in leprosy and internally for ancylostomiasis by the local people in South Africa (Watt & Breyer-Brandwijk 1962; Bryant 1966; Khan *et al.* 1978) It was therefore, decided to investigate the inhibitory activity of acetone and water extracts of *E. natalensis* and an isolated compound, diospyrin, against herpes simplex virus type-1 (HSV-1) *in vitro* on primary vervet monkey kidney cells.

Cell toxicity was monitored by determining the effect of the extracts and diospyrin on the monolayers of VK cells. The dose of the plant samples (ID_{50}) that inhibited 50% cell growth after the incubation period was found to be 0.1, 0.2 and 0.02 mg/ml for acetone, water extracts and diospyrin respectively. Water extract from the roots of the plant was the least toxic to cell cultures and moderately inhibited the replication of HSV-1 at a concentration of 0.2 mg/ml. Diospyrin had no inhibitory effect against the virus however, the acetone extract inhibited the replication of the virus at concentrations ranging from 0.1 to 0.02 mg/ml as shown by the reduction of virus induced cytopathogenic effect and the protection of cells in MTT assay (3-4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide).

9.7 References

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