Variation of active constituents in *Euclea natalensis* based on seedling stages, seasons, and fertilizers

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BY

DECLARATION

I the undersigned, declare that these studies, except where acknowledged in the text, is my own work and has not been previously submitted in any other form to this or to any other institution.

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Abstract

Euclea natalensis A.DC. belongs to the Ebenaceae family, and is extensively distributed along the eastern coast of southern Africa. Many *Euclea* species are widely gathered by indigenous people because of their medicinal properties. Roots of these plant species are frequently used to treat respiratory complications such as chest pains, bronchitis, pleurisy and asthma. Ground root powder is topically applied in cases of leprosy and is used by some ethnic groups to treat toothache and headache. The bioactivity encountered is attributable to naphthoquinones, which are common phenolic compounds in the Ebenaceae family. Naphthoquinones isolated from *E. natalensis* (shinanolone, 7-methyljuglone, diospyrin, isodiospyrin and neodiospyrin) have exhibited a broad spectrum of antimicrobial activity. The demand for these products will escalate due the amount of plant material required to further research. We need to explore techniques that can maximize their productivity. The present study was conducted on *E. natalensis*, in an attempt to establish if there exists any correlation between the accumulation of naphthoquinones and stages of seedling growth, seasonal fluctuations and application of fertilizers.

A possible correlation between seedling growth stages and the accumulation of naphthoquinones (shinanolone, 7-methyljuglone and diospyrin) was investigated in seeds and seedlings of *Euclea natalensis*. In this study, the seeds represented the first stage, whereas the second seedling stage was defined as the stage when the radicles were about 6 cm long. The lengths of the seedlings at the third, fourth and fifth

seedling stages were 9 cm, 12 cm and 16 cm respectively. Plant materials collected from the five seedling stages were separately extracted using chloroform and the naphthoquinones were then quantified by means of High Performance Liquid Chromatography (HPLC). Mobile phase of MeCN: H_2O: AcOH (62.5: 32.5: 5) was used as an eluent in an isocratic mode and at a flow rate of 0.8 ml/min. Standard curves of each of the four compounds were obtained by making a series of dilutions in the concentration range of 22.5 µg/ml to 2.25 µg/ml. Ten microlitres of each dilution was injected three times into the HPLC, and the run time for each injection was 20 minutes. Calibration curves were then generated and used for the quantification of each compound.

Shinanolone, which was the only naphthoquinone detectible in seeds, accumulated at variable rates ($P<0.01$) and no trend could be established between its synthesis and seedling growth. The content of shinanolone ranged from 87.5 mg/kg dry weight (dw) in seeds to a high mean value of 1047 mg/kg (dw) during the fourth seedling stage. A significant correlation ($P<0.01$) was found between the mean concentrations of 7-methyljuglone and seedling growth. 7-Methyljuglone was quantified at a high mean level of 5003 mg/kg during the third seedling stage and was not detected in the seed samples. A positive correlation ($P<0.01$) was established between the concentration of diospyrin and seedling stages. Diospyrin was detected at an elevated mean concentration of 6182 mg/kg during the fifth seedling stage, which was higher than the other quantified naphthoquinones.

Seasonal variation of naphthoquinones (shinanolone, 7-methyljuglone, diospyrin, isodiospyrin and neodiospyrin) was investigated from eleven plants of *E. natalensis* subsp. *natalensis* growing in natural populations, over a period of four seasons. The roots were harvested, dried, extracted and analysed as in the previous study. The mean

levels of shinanolone and 7-methyljuglone were found to be uniform in all the seasons and no statistically significant variation could be found between seasonal changes and their mean concentrations. Accumulation of isodiospyrin and neodiospyrin varied significantly with seasonal changes ($P<0.05$). These two bioactive naphthoquinones were detected only in summer and autumn respectively, and not in winter. A statistically significant variation ($P<0.05$) was established between the levels of diospyrin and seasonal fluctuations. Diospyrin was detected at a mean concentration of 3190 mg/kg (dw) during spring, which was higher than the other naphthoquinones quantified in all four seasons.

The effect of NPK fertilizers on growth performance and accumulation of naphthoquinones (shinanolone, 7-methyljuglone, diospyrin, isodiospyrin and neodiospyrin) in seedlings of *E. natalensis* grown in shade and under field conditions was investigated. Each group was subdivided into four subgroups, which were then subjected to four respective treatments of water-soluble foliar feed (2:1:2 (44) NPK) at three different concentrations. Treatments tested were as follows: Treatment 1 at 40 g/l, Treatment 2 and Treatment 3 at 20 g/l and 10 g/l respectively. The control group received only supplemental water. The first harvest was conducted after 6 months of application of fertilizers and the second one was done after 12 months of treatment. Roots and shoots were harvested and analysed separately. The naphthoquinones were quantified as previously described. The bioactivity of root extracts from seedlings was tested against *Mycobacterium smegmatis* and extracts with lower MIC were further tested on *M. tuberculosis.*

Growth parameters differed between the two groups, with seedlings from the shadehouse showing more plant vigour than the field grown plants. No significant interaction could be established between the measured growth factors and treatment.

A significant interaction ($P<0.001$) was found between Treatment 2 and shadehouse seedlings. Treatment 2 enhanced vegetative performance with the mean values of fresh weight of shoots and roots being twice as much as their respective control mean values. A significantly positive correlation was established between the concentration of shinanolone ($P<0.01$), isodiospyrin ($P<0.05$) and neodiospyrin ($P<0.05$) with fertilization from field-grown seedlings. Application of NPK fertilizers significantly ($P<0.05$) increased the accumulation of neodiospyrin in seedlings subjected to shadehouse conditions. The most potent naphthoquinone, 7-methyljuglone, was found to be abundant in all the extracts and was quantified at a high mean concentration of 10200 mg/kg from shadehouse seedlings.

Root extracts of *E. natalensis* seedlings grown under field conditions were generally more active against the bacterial strain of *M. smegmatis* as compared to extracts acquired from roots of seedlings maintained under a shadehouse setting. A lowest minimum inhibitory concentration (MIC) of 0.78mg/ml against *M. smegmatis* was observed from the second harvest of field-cultivated seedlings of the control and Treatment 1 subgroups. The MIC values for shadehouse seedlings ranged from 1.6 to 6.3 mg/ml. Minimum bactericidal concentration (MBC) values from all the extracts tested were relatively higher than their respective MIC's. Root extracts of *E. natalensis* were more active against *M. tuberculosis* and their MIC values were lower than the tested concentrations. Extracts acquired from field-grown seedlings were more active against *M. smegmatis* with a lowest MIC value of 0.78 mg/ml. Extracts from the control group and Treatment 1, which had less application of fertilizers were more active against strains of *M. tuberculosis* with MIC value of 10 µg/ml. This shows the selectivity of *E. natalensis* against the mycobacterial strain of *M. tuberculosis*.

Based on the findings, synthesis and accumulation of naphthoquinones in *E. natalensis* is highly variable within individuals of the species investigated. Naphthoquinones accumulate in relatively higher amounts in roots of *E. natalensis* than in the aboveground structures, which validate their harvest by indigenous people. The concentration of shinanolone varied slightly and its production increased with seedling growth. The synthesis of 7-methyljuglone is independent of fertilisation as its accumulation was enhanced in seedlings subjected to control treatment. Neodiospyrin and isodiospyrin were always present in every sample obtained from the seedlings but they were not detectible in every profile of samples from mature plants. Diospyrin is the only naphthoquinone that was detected in every sample analysed and also quantified in high concentrations from mature plants harvested in spring. The study showed that depending on the requirement of a particular naphthoquinone for research, one could target the seasons and seedling stages recommended from this study. This study also showed that field-cultivated seedlings produced more potent naphthoquinones than the ones subjected to controlled environments.

CONTENTS

LIST OF ABBREVIATIONS

ANOVA	**-**	**analysis of variance**
AcOH	**-**	**acetic acid**
BACTET		**Becton Dickinson Diagnostic Instrument**
CFU		**colony forming units**
DMSO		**dimethyl sulfoxide**
GPS		**global positioning system**
HPLC	**-**	**high performance liquid chromatography**
GI		**growth index**
INH		***p*-iodonitrotetrazolium chloride**
MBC		**minimum bactericidal concentration**
MeCN	**-**	**acetonitrile**
MIC		**minimum inhibitory concentration**
NPK	**-**	**nitrogen: phosphorus: potassium**
OADC		**oleic albumin dextrose catalase**
RCBD	**-**	**random complete block design**
SAS	**-**	**statistical analysis system**
TB	**-**	**tuberculosis**
TLC	**-**	**thin layer chromatography**
UV	**-**	**ultraviolet**

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CHAPTER 1

Introduction

1.1. Problem statement

The current resurgence of interest in screening medicinal plants is primarily aimed at discovering novel secondary metabolites, in response to the unprecedented prevalence of resistant microbial strains, that are causing diseases and infections of epidemic proportions (Nigro *et al*., 2004). These pathogens are evolving at a faster rate, far outstripping and undermining treatment by drugs, that are currently indispensable for preventing progression of diseases. Medicinal plants have provided a wide variety of secondary products with diverse structures and biological activities, many of which have found therapeutic applications in the health sciences (Gao *et al*., 2001). Regardless of the advances that are made with synthetic products as alternative supply of bioactive compounds, there is a growing global trend towards herbal medicines. Apart from showing fewer side effects, plant-derived secondary compounds serve as prototypes in the modulation of chemotherapeutic agents.

Plants synthesize and accumulate secondary metabolites in relatively trace amounts and cannot meet the escalating demand in pharmaceutical drug development. In some cases the active principle is present in minute quantities in the plant extract and therefore, cannot be used for clinical trials that are important steps required in validating the bioactivity of the compound of interest. One other common problem associated with obtaining plant products from natural resources is the variation in the concentration of secondary compounds due to seasonal fluctuations, nutrient status and developmental stages (Liu *et al*., 1998; Elgorashi *et al*., 2002; Laitinen *et al*., 2005). In some instances the desired plant species are located in politically unstable regions, which can be a major drawback to progress in research. The

screening of medicinal plants for drug development needs to be conducted in parallel with studies that involve optimization of secondary compounds through chemical synthesis, plant breeding and biotechnological techniques. An understanding of the synthesis and accumulation of secondary metabolites in plants growing in wild settings is also required. These investigations and other related studies would ensure the continual supply of plant-derived materials without diminishing our natural resources.

Euclea natalensis was selected as a model plant system for this research because of its medicinal potential, which is commonly ascribed to the presence of naphthoquinones. Several naphthoquinones including shinanolone, 7-methyljuglone, diospyrin, isodiospyrin and neodiospyrin have been isolated from *E. natalensis* (Ferreira *et al*., 1977). Previous research involved studies such as antimycobacterial activity of crude extracts from *E. natalensis* (Lall and Meyer, 1999), inhibition of *Mycobacterium tuberculosis* by diospyrin (Lall and Meyer, 2001), antimycobacterial activity of diospyrin derivatives (Lall *et al*., 2003) and characterization of intracellular activity of constituents from roots of *E. natalensis* (Lall *et al*., 2005).

An intensive investigation is currently underway in an attempt to characterize the antimycobacterial compounds that have shown significant activity against drug-sensitive and drug-resistant strains of *M. tuberculosis.* Studies are presently taking place in partnership with other departments and medical research institutions to further explore the medicinal and other biochemical properties of the naphthoquinones isolated from *E. natalensis*. Several experiments such as synergistic trials with existing TB-drugs, chemical synthesis of bioactive naphthoquinones as well as their derivatives and their *in vivo* tests are in progress and other significant research on *E. natalensis* is anticipated.

During the studies, difficulties were encountered in acquiring certain active metabolites

from the roots of *E. natalensis*, which were collected during different seasons. This prompted a study to evaluate possible seasonal fluctuations in the accumulation of naphthoquinones. It was also decided to determine their concentrations at various stages of seedling growth of *E. natalensis*, maintained under uniform conditions. The effect of fertilizers on the synthesis and accumulation of naphthoquinones in seedlings of *E. natalensis*, cultivated under field conditions and in greenhouse settings, was also examined. The bioactivity of crude extracts from seedlings grown in shade and under field conditions was then evaluated on strains of *Mycobacterium smegmatis* and *M. tuberculosis*. The study is not directly involved in the antimicrobial tests and clinical trials that could lead to drug development, but it can be used to aim the appropriate time of harvest for increased yields of naphthoquinones. Cultivated *E. natalensis* seedlings can also be used to replace the plant material harvested from natural habitats.

1.2. Objectives

The specific objectives of this research were to:

- Investigate the variability in the content of naphthoquinones in seeds and various seedling stages of *E. natalensis*.

- Examine the seasonal variation of naphthoquinone accumulation in roots of *E. natalensis*.

- Evaluate the effect of NPK fertilizers on growth and naphthoquinone production

in seedlings of *E. natalensis*.

1.3. Scope of study

Chapters 1, 2, 3, 4 and 6 have been written according to the format of the South African Journal of Botany. Chapter 5 has been written in a format that conforms to the structure of Journal of Natural Product Research for peer-review and therefore, a level of inconsistency will be encountered in the thesis.

Chapter 2 This chapter outlines a literature review on the qualitative and quantitative variation of secondary metabolites within and among plant species. It also presents various techniques that are currently applied to enhance the production of bioactive compounds of medicinal plants.

Chapter 3 The content of naphthoquinones in seeds and seedlings of *E. natalensis* were quantified, so as to establish if there is any significant variation in their accumulation in relation to seedling growth.

Chapter 4 Variation of naphthoquinones as a function of seasonal changes was investigated in root samples harvested over four seasons from a population of *Euclea natalensis* subspecies *natalensis.* The levels of the four naphthoquinones under investigation were then quantified, in an attempt to establish a correlation between their production and seasonal

changes.

Chapter 5 Seedlings of *Euclea natalensis* were subjected to differential treatments of NPK fertilizers, so as to evaluate the effect of fertilizers on the production and accumulation of naphthoquinones. The vegetative performance of the seedlings was also observed.

Chapter 6 General interpretations based on the findings of the research conducted are presented in this chapter and suggestions for future research are also highlighted.

Chapter 7 Conference presentations and manuscripts based on the present study have been mentioned.

Chapter 8 Acknowledgements and credits are given to those who made contributions towards the research undertaken.

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CHAPTER 2

Literature review

2.1. Phytochemical variation

The continued reliance of the human species on medicinal plants is an evident reflection of the long history of people-plant interactions that has been significantly enhanced by human development (Nigro *et al*., 2004). Along their cultural diversification, indigenous people acquired a wealth of information on the curative potential of plants growing within their immediate environments. The traditional knowledge was then passed on from generation to generation and plants continued to be the major source of medicines to most human beings. Apart from the acquisition of knowledge on medicinal properties that plants are endowed with, natives have also developed an understanding of patterns of variation in the effect of herbal remedies.

The variations observed are direct indices of variables such as diurnal factors, seasonal fluctuations, developmental stages, level of pathogens, geographical differences and the nutrient status of the soil (see for example, Itenov *et al*., 1999; Agerbirk *et al*., 2001; Castells *et al*., 2005; Qu *et al*., 2005). These factors may exert trade-offs between plant growth and accumulation of secondary compounds. Being firmly rooted in the ground, plants cannot move to escape harsh environmental conditions or the unwanted attention of animals and microorganisms that depend on them for their survival. In order to maximize fitness for survival, plants divert and allocate resources to the synthesis of secondary metabolites, which act as chemical mediators in the antagonistic interactions between plants and their environment.

Despite the fact that high concentrations of secondary metabolites might result in a relatively resistant plant, their production is thought to induce a fitness cost that may reduce plant growth and reproduction (Van der Meijden *et al*., 1988; Siemens *et al*., 2002). The

synthesis of secondary compounds is costly to the plant because it requires continuous flow of precursors from primary metabolism to drive their biosynthetic pathways. The amount of cost depends on the nature of the secondary metabolite, its biosynthetic origin and availability of resources required for its production (Han and Lincoln, 1994). Close and McArthur (2002) argue that under low nutrient environments light enhances photosynthetic rates and cheap carbon will be available and allocated to the synthesis of carbon based secondary compounds such as phenolic compounds. If nutrients are abundant and light is limited, plants will increase the production of nitrogen-based compounds such as alkaloids (Behmer *et al*., 2002; Lerdau, 2002).

Higher plants vary in resource allocation patterns, which occur through differences in chemical composition of structures, the relative mass of different structures or organs and relative number of different structures a plant produces (Bazzaz *et al*., 1987). The allocation pattern of a plant defines its ecological roles and is therefore an important factor in understanding the influence of environmental variables on the synthesis and accumulation of secondary compounds. Knowledge on how plants allocate resources would enhance our ability to characterise and predict biosynthetic patterns of secondary metabolites in wild settings and to further apply strategies that will maximize their production in laboratory settings and under field conditions.

2.2. Optimization of phytochemicals

It is well established that some rural and tribal people have a direct dependence on medicinal plant collection and sale for their livelihood. Many medicinal plants are being exploited and that is continuously exerting pressure on our natural resources. Human population growth, industrial applications of secondary plant products and the current outbreak of drug resistant microbes are some of the factors exacerbating the present over-utilization of natural resources (Tally, 1999). Plants are the source of many modern medicines and many prescribed drugs contain plant extracts or active ingredients obtained from or modelled on plant products (Newman *et al*., 2003). The quest for screening medicinal plants is unavoidable. The long-term effects of these injudicious harvests of medicinal plants may lead to the extinction of valuable plant species.

Plants synthesize and accumulate secondary compounds in relatively small amounts and cannot meet the increasing demand in the pharmaceutical industry and herbal trades. In most cases the active principle is present in minute quantities in the crude extract and therefore, may not be enough to further clinical trials that are important steps required in validating the bioactivity of the compound of interest. There is a growing trend towards optimization and enhancement of phytopharmaceuticals in an attempt to redress their shortage and overexploitation of wild plant populations (Reinten and Coetzee, 2002; Nigro *et al*., 2004). Various approaches have evolved so as to enhance the production of natural products. These include synthetic compounds, large-scale cultivation of medicinal plants and optimization through biotechnology.

2.2.1. Chemical synthesis of secondary metabolites

Natural products have a long history of providing valuable drug leads for the production of a broad variety of indispensable industrial pharmaceuticals (Leventine and McMahon, 2003). They represent a source of molecular diversity for drug discovery and development; furthermore they are also complementary to other molecular sources such as high-throughput synthesis and combinatorial libraries (Shu, 1998). While the value of screening natural products extracts is indisputable, it is also associated with several shortcomings. Building up and maintaining high-quality natural products is expensive and time-consuming (Ortholand and Ganesan, 2004). The separation of the target compound may be difficult and a level of inconsistency is also encountered depending on seasons during which plant collection is carried out (Kutney, 1998). The synthetic approach may provide a less expensive source of secondary compounds. Such analogues can be obtained through a single route that is feasible and economical, as compared to the conventional way of isolating plant products (Van Der Kooy and Meyer, 2006).

Some of the pharmaceutical industries, whose product portfolios are predominantly based on synthetic drugs, screen and then model their final products based on the molecular structures of natural products isolated from plant sources (Shu, 1998; Newman *et al.*, 2003). Advances in organic and analytical chemistry have enabled chemists to design molecules that resemble natural products in their biochemical qualities. Chemical synthesis through introduction, alteration or removal of a functional group, permits variations in the structure, which may lead to optimization of antimicrobial and pharmacokinetic properties (Seimiya *et al.*, 2002; Zhang *et al.*, 2006). These chemical modifications may lead to a wide spectrum of functionally different substances with enhanced pharmacological activities such as bioavailability, selectivity and binding affinity of the synthetic compound.

Although the synthetic approach appears to be a commercially viable route, there is an increasing preference for natural products. These renewed interests in herbal remedies are in response to the disadvantages that are often associated with purely synthetic medicines. The therapeutic effectiveness of synthetic medicines is easily circumvented by pathogens due to the failure of chemists to synthesize compounds that can employ different chemical strategies for the control of microbial infections (Lewis and Ausubel, 2006). Formulation of synthetic medicines usually involves the use of inorganic compounds and the reagents in excess or reaction by-products, may result in undesired chemical interferences with side-branches of the synthesised compound. While the safety and efficacy of a drug molecule is largely dependent on its chemical structure and not its origin, the purification of synthetic chemicals is not always 100 % efficient. Remains from their formulation vehicle can have long-term residual activity in the biochemical environment of living organisms and that may cause side effects.

Development of drugs and the subsequent pharmacological enhancement, require knowledge of the specific molecular target of the drug, such as a receptor or enzyme. It takes years to learn what approaches can provide some benefits and to progress to a point where the disease is effectively managed with medicines devoid of side effects (Gibbs, 2000). It is also difficult to derive a clinically useful drug from complex natural products that are under the control of several genes. Such synthetic routes entail conversions of several complex intermediates to the desired products and therefore, require multi-step synthesis, which can be tedious and expensive (Kutney, 1998). Anticancer agents such as paclitaxel (Fig. 2.1) and adriamycin (Fig. 2.2) are difficult to make synthetically and are still obtained from natural sources such as *Taxus brevifolia* and *Streptomyces peucetius*.

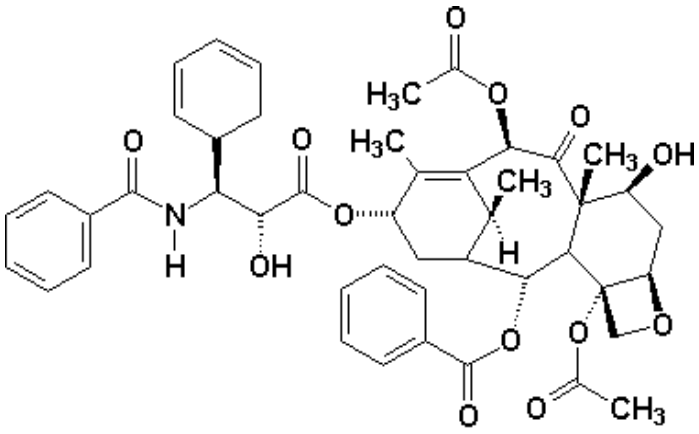


Figure 2.1. Structure of paclitaxel. (www.rxlist.co.za)

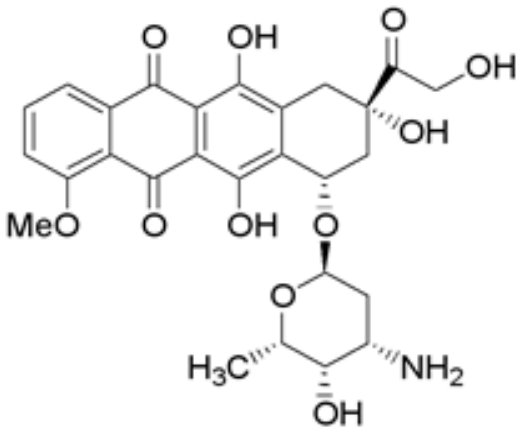


Figure 2.2. Structure of adriamycin. (www.it.monline.org)

2.2.2. Cultivation of medicinal plants

The consequences of the present unscrupulous utilization of natural resources are posing a serious threat to our biodiversity and have become a major concern underlying the concept of sustainable development. More and more medicinal plants are disappearing

owing to their commercialization that is leading to land degradation and instability among wild populations (Mander *et al*., 1996; Williams *et al*., 2000). According to Arnold *et al*. (2002), 86 medicinal plant species are Red Data Listed and 4% of the nearly 4000 medicinal plants in southern Africa are threatened, which is negatively affecting the medicinal plant taxa. It is becoming increasingly difficult to acquire plant-derived components from wild stocks, due to injudicious harvest by gatherers, that is leading to a rapid depletion of natural plant populations. Socio-economic constraints are the central forces driving the overexploitation of plants endowed with medicinal properties.

The phytopharmaceutical value of these species and their continual use in traditional medicine are escalating their demand, which cannot be met by sustainable harvesting alone. It has become inevitable to guarantee a continuous supply of renewable resources of raw plant materials to both local and global markets through effective propagation and breeding methods (Jäger and Van Staden, 2000; Reinten and Coetzee, 2002). Threatened and rare species can be cultivated on a large scale, some of which can be reintroduced to their wild habitats for restoration and protection of ecosystems (Smith *et al*., 2002). *Warburgia salutaris* (Fig. 2.3), which was listed as vulnerable and locally extinct due to overharvesting for medicinal purposes, has been successfully reintroduced through transplantation to its natural habitats in southeastern Zimbabwe (Cunningham, 2001). Its reintroduction was considered useful from a conservation perspective, because of its overexploitation by street vendors. Indigenous Zimbabweans use its bark to cure common colds, malaria and mouth sores.

Domestication and development of wild species with medicinal properties as cash crops, will also boost the natural products subsector. It will enhance its capacity to contribute to the economic growth among rural entrepreneurs, thereby generating employment and hence

Figure 2.3. ***Warburgia salutaris*** **(www.plantzafrica.com).**

sustainability of livelihoods (Appleton and Van Staden, 1995; Evans and Sengdala, 2002). Cultivated medicinal plants have shown some pharmacological advantages over the wild collected ones. Wild harvested plants usually vary in quality and composition due to environmental and genetic differences, which can result in uncertainty of their respective therapeutic benefits. Cultivated plants are maintained under uniform conditions for standardization of the active constituents. They can be irrigated for increased yields and harvested at the appropriate time (see, for example, Abrie and Van Staden, 2001; Kothari and Singh, 2003).

The transition from wild settings into field trials and the eventual crop development is fraught with practical limitations that can retard the process of commercialization. Some of the wild species are not readily adaptable to domestication due to lack of environmental

cues that are normally part of their natural habitats. Their growth characteristics are often unsuitable for cultivation as most of them are difficult to propagate through vegetative reproduction. Seeds of some wild species display a deep dormancy with a high level of asynchronous germination, resulting in seedlings that are not morphologically uniform. Jäger and Van Staden (2000) cautioned that harvesting seeds from wild populations would deplete seed banks and hence decrease their gene pool.

There are numerous micropropagation protocols that have been established for several South African medicinal plants including *Hypoxis* and *Scilla* species (Appleton and Van Staden, 1995; Hanneweg *et al*., 1996). Some regenerated plantlets are not amenable to the culture conditions and show less resilience in the wake of pathogenic infections because of their genetic uniformity (Chakravaty and Sen, 1989). Generally, plants synthesize secondary metabolites in response to the pressure exerted by both biotic and abiotic ecological factors, found within their immediate environment, and if such interactions are broken, then they are most likely to yield trace amounts of the compounds of interest. Providing plants with optimal resources and favourable environmental conditions can induce shifts in their allocation patterns, resulting in unusual trade-offs between their agronomical traits and chemical qualities (Máthé, 1988). Furthermore the quality of the plant products needs to be systematically evaluated from each harvest for their efficacy and validity, which can be lengthy, costly and delaying the supply of plant products.

2.2.3. Biotechnological applications

Biotechnology is gaining more preference over the other conventional methods as potential tools to enhance the production of secondary metabolites. They include micropropagation using callus (Fig. 2.4.), plant cell suspension, root, embryo and protoplast cultures. Undifferentiated cells or tissues are grown under suitable conditions to yield large quantities of biomass that can synthesize large amounts of valuable secondary

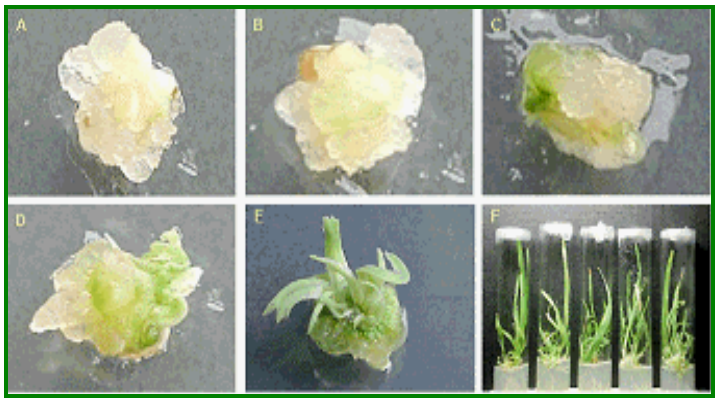


Figure 2.4. Different stages of plant tissue culture. Three week-old calli (A). One day-old initiated shoots (B). Three day-old initiated shoots (C). Six day-old initiated shoots (D). Ten day-old initiated shoots (E). Plantlets (F). (www.cropscience.org).

metabolites (Tripathi and Tripathi, 2003). Due to the lack of some physiological traits responsible for secondary metabolism in undifferentiated cells, *in vitro* production of secondary compounds from differentiated cultures is often chosen, so as to retain the biosynthetic characteristics of the original plant. Elicitors such as salicylic acid are frequently applied to the cultures in order to trigger the *de novo* synthesis of secondary metabolites (Mulabagal and Tsay, 2004).

Genetic manipulation of *in vitro* cultures such as the hairy root transformation are used to increase the biosynthetic activity and thus enhancing the level of product yield (Hirsikorpi *et al*., 2002; Hu and Du, 2006). These genetic transformation techniques allow for the manipulation of secondary metabolic pathways in whole-plant, as well as in tissue and cell cultures. Proliferation of high producing lines can result in large amounts of biosynthetically active tissues under controlled environments, in which seasonality, age specificity and negative biological influences would be circumvented (Mulabagal and Tsay, 2004). Direct and indirect methods of gene transfer are being used in transformation studies with medicinal plants with the intent of optimising secondary metabolite production (Verpoorte and Memelink, 2002; Nigro *et al*., 2004).

Although the biotechnological approach to optimisation of plant products appears to be a more viable option, there are many limitations associated with it. In practice, many woody species are recalcitrant and not amenable to the *in vitro* culture conditions, which is one of the major obstacles to successful regeneration of cells, tissues, organs and whole plants. Some cultures lack the desired biosynthetic activity or yield relatively low concentrations of secondary metabolites, due to the absence of specialized structures such as trichomes, fruit capsules and laticifers in which products accumulate (Franke *et al*., 2002). Gao *et al*. (2001) found that the contents of main active saikosaponins in adventitious roots of *Bupleurum falcutum* cultured in bioreactors were almost the same as those in roots of field cultured plants.

Production of unusual and non-targeted compounds is also a common feature of *in vitro* cultures, which may be ascribed to mutagenic agents, low levels of expression of key enzymes at rate limiting steps in a pathway, or uncoordinated fragmentary expression of genes. Partial operation of a given pathway can lead to low and variable production of

secondary products. This requires investigation on the molecular regulation of biosynthetic pathways and the expression of regulatory genes that control multiple biosynthesis (Verpoorte and Memelink, 2002). Empirical studies on manipulation of the expression of desired secondary pathways are not yet sufficient and there is a compelling need to integrate knowledge of the enzymology and molecular biology of these pathways. In addition to the costly equipment necessary to conduct these studies, trained engineers are also few and all these can place major financial constraints on developing countries such as South Africa.

Although there are a number of disadvantages associated with the above techniques, they can still offer an option to the exploitation of our natural resources. Some of these techniques will be efficient in some plant species, resulting in maximum yields of compounds that can be used in industrial applications. In cases where a particular optimization system is inefficient for a given plant species, other methods of enhancement should be explored. This should be conducted systematically so as to develop a protocol that can later be used by other researchers for related plant species. A thorough comparative analysis between different techniques should also be considered, so as to lower the risk-benefit ratio that is usually encountered in many institutions.

2.3. *Euclea natalensis*

Euclea natalensis (Fig. 2.5.) belongs to the family Ebenaceae and is distributed in a wide range of habitats and also forms part of some bushveld communities (Van Wyk and Van Wyk, 1997). It is widely distributed along the eastern coast of southern Africa (Fig. 2.6.),

inland forests of Botswana and Swaziland (Ms. Snyman, National Botanical Institute, Pretoria, 2004). It is an evergreen, woody plant species, that grows as a shrub to a medium sized tree of about 12 m in height, and produces red indehiscent berries (Palgrave, 1991; Van Wyk and Van Wyk, 1997). Indigenous people of southern Africa harvest the roots of *E. natalensis* for a number of medicinal applications. The Zulus use its root bark to treat respiratory complications such as chest pains, bronchitis, pleurisy and asthma. Shangaans apply the ground powder to the skin in cases of leprosy, and the ground plant material is also used by many ethnic groups of southern Africa to relieve headache and toothache (Bryant, 1966).

Figure 2.5. *Euclea natalensis* (Van Wyk and Van Wyk, 1997).

The antimicrobial activity of *E. natalensis* is attributable to the presence of naphthoquinones (see Fig. 2.7.) among other compounds, which are the dominant secondary metabolites found within the Ebenaceae family (Mallavadhani *et al*., 1998; Mebe *et al*., 1998; Khan and Timi, 1999; Van Wyk and Gericke, 2000). Naphthoquinones

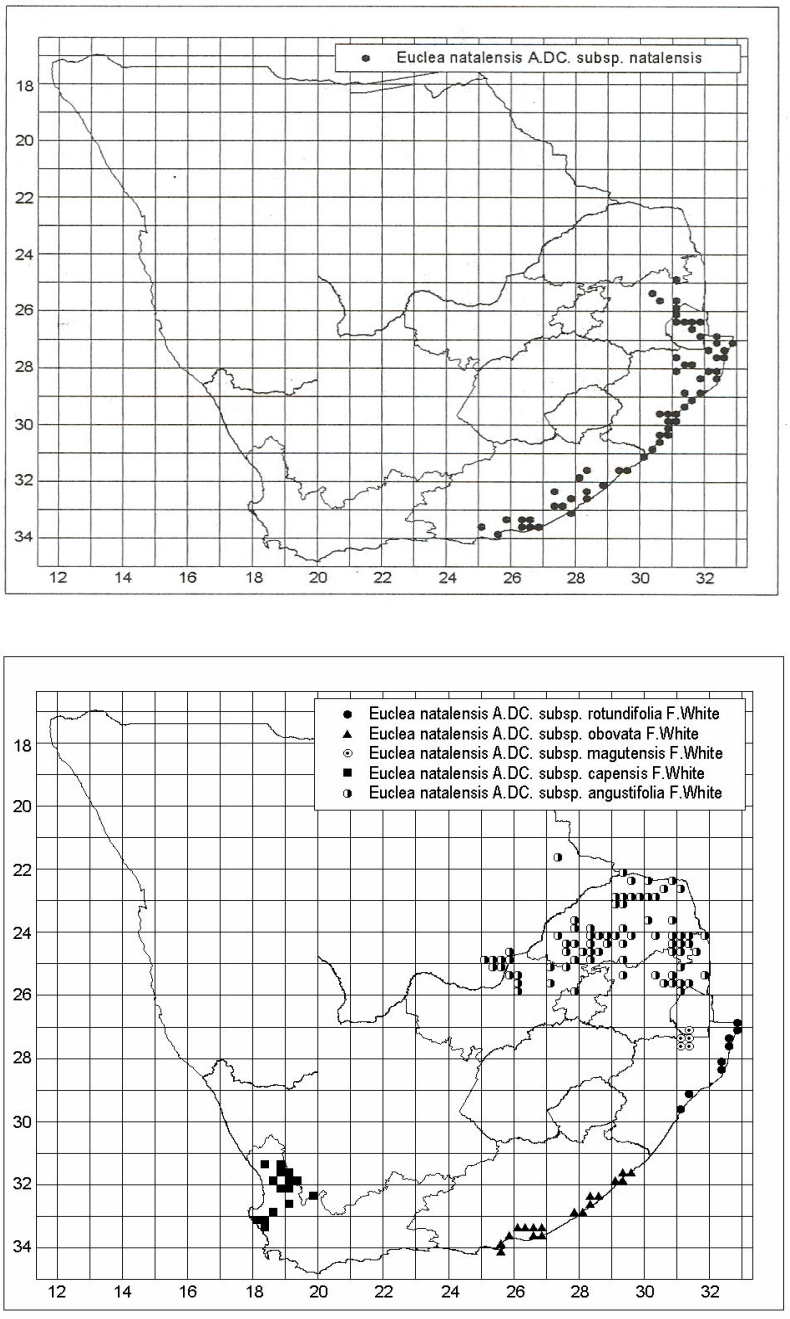
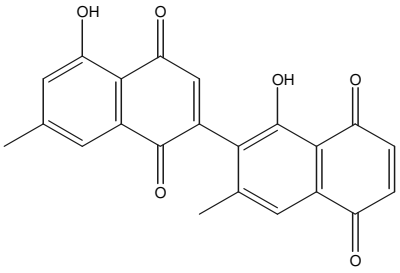


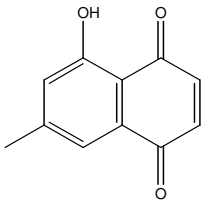
Figure 2.6. Distribution of the subspecies of *Euclea natalensis* in southern Africa (NBI, 2004).

are widely reported to act in allelochemical modes (Ohno, 2001), functioning as subterranean signal molecules in the rhizosphere (Wrobel *et al*., 2002) and inhibiting growth from a wide variety of microorganisms such as larval and fungal pathogens (Sasaki *et al*., 2002). Tokunaga *et al*. (2004) reported on their mechanism of antifeedant activity against predators of the Droseraceae family. These properties endow them with the ability to confer bioactivity against a wide spectrum of microbes. Their medicinal potential is however, limited by their cytotoxicity and low bioavailability (Gafner *et al*., 1987; Kayser *et al*., 2000). In addition to their ecological and functional attributes, naphthoquinones have found widespread applications in the dye industry as well as photostabilizers (Couladouros *et al*., 1996).

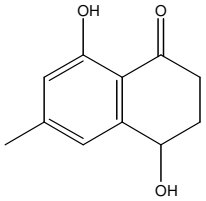
Phytochemical analysis of crude extracts from the roots of *E. natalensis* revealed the presence of 7-methyljuglone and its dimers diospyrin, isodiospyrin, neodiospyrin, mamegakinone, as well as shinanolone and pentacyclic triterpenoids (Fig. 2.7.), all of which were previously isolated (Orzalesi *et al*., 1970; Van der Vijver and Gerritsma, 1973; Ferreira *et al*., 1977; Khan, 1985; Mebe *et al*., 1998). Several antimicrobial studies have been conducted to evaluate the pharmacological efficacy of naphthoquinones. Isodiospyrin has been examined for its inhibition of human DNA topoisomerase I (Ting *et al*., 2003), antibacterial (Adeniyi *et al*., 2000) and anti-inflammatory (Kuke *et al*., 1998) activities. 7-Methyljuglone is well documented for its antibacterial activity against oral pathogens (Cai *et al*., 2000) and was also shown to be toxic to wood termites (Carter *et al*., 1978). Due to their cytotoxic properties, many naphthoquinones have shown inhibitory activities on different cancer cell lines (Gafner *et al*., 1987; Kapadia *et al*., 1997; Hazra *et al*., 2005; Wube *et al*., 2005). A number of naphthoquinones have been well reported to show antiprotozoal and antifungal activities (Ioset *et al*., 1998; Kayser *et al*., 2000; Sasaki *et al*.,



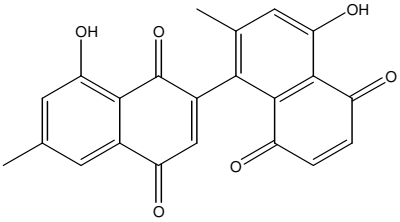
diospyrin



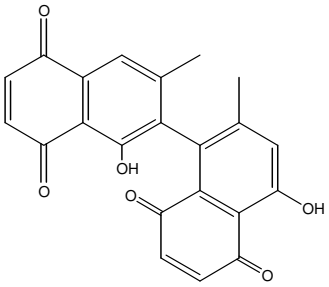
7-methyljuglone



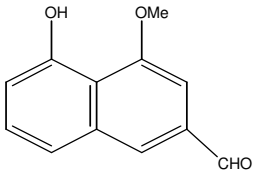
shinanolone



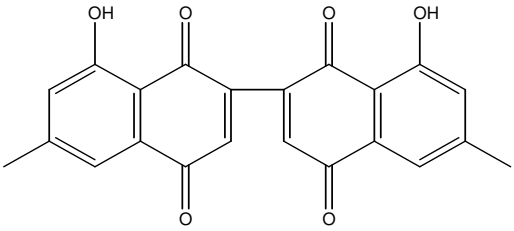
neodiospyrin



isodiospyrin



5-hydroxy-4-methoxy-2-naphthaldehyde



mamegakinone

Figure 2.7. Naphthoquinones isolated from *Euclea natalensis*

2002). Crude extracts and naphthoquinones isolated from *E. natalensis* were tested on a number of bacterial strains (Khan *et al*., 1978), especially on *Mycobacterium tuberculosis* (Lall and Meyer, 1999; Lall and Meyer, 2001; Lall *et al*., 2003; Lall *et al*., 2005) on the basis of its ethnomedical use by natives of southern Africa.

The medicinal potential of *E. natalensis* is not fully explored and more bioactive naphthoquinones are required for further research. Acquisition of some naphthoquinones is at times difficult, due to an uneven accumulation of these plant products by *E. natalensis*. The present study was undertaken in an attempt to evaluate the feasibility of applying some methods and techniques that will maximize the availability of naphthoquinones.

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CHAPTER 3

Variation in the content of naphthoquinones in seeds and seedlings of *Euclea natalensis*

#Variation in the content of naphthoquinones in seeds and seedlings of *Euclea natalensis*

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3.1. Abstract

A correlation between plant growth and accumulation of naphthoquinones (shinanolone **(1)**, 7-methyljuglone **(2)** and diospyrin **(3)**) was investigated in seeds and seedlings of *Euclea natalensis* A.DC. In this study, the seeds represented the first stage whereas the second seedling stage was defined as the stage, when the radicles were about 6 cm in length. The lengths of the seedlings at the third, fourth and fifth seedling stages were 9 cm, 12 cm and 16 cm respectively. Plant materials collected from the five seedling stages were separately extracted using chloroform and the naphthoquinones were then quantified by means of High Performance Liquid Chromatography (HPLC). Shinanolone **(1)**, which was the only naphthoquinone detectible from the seeds, accumulated at variable rates ($P<0.01$) and no trend could be established between its synthesis and seedling growth. The content of shinanolone **(1)** ranged from 87.5 mg/kg in seeds (first stage) to a high mean value of 1047 mg/kg during the fourth seedling stage. A significant correlation ($P<0.01$) was found between the mean concentrations of 7-methyljuglone **(2)** and seedling growth. 7-Methyljuglone **(2)** was quantified at a high mean level of 5003 mg/kg during the third

seedling stage and was not detected from the seed samples. A positive correlation ($P<0.01$) was established between the concentration of diospyrin **(3)** and seedling stages. Diospyrin **(3)** was detected at an elevated mean concentration of 6182 mg/kg during the fifth seedling stage, which was higher than the other quantified naphthoquinones.

Keywords: Ebenaceae; *Euclea natalensis*; Naphthoquinones

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3.2. Introduction

Euclea natalensis A.DC., a member of the Ebenaceae family, is extensively distributed along the eastern coast of southern Africa, extending inland into Swaziland and Botswana (Van Wyk and Gericke, 2000). It grows as a shrub to a medium sized tree of about 12 m in height, often with a spreading crown and occurs in a wide variety of habitats (Palgrave, 1991). Southern African natives use the roots of *E. natalensis* for a number of medicinal purposes. These include the preparation of purgatives, topical application to skin lesions in cases of leprosy (Palgrave, 1991), relief of toothache, headache and chest complaints (Van Wyk and Gericke, 2000). The pharmacological activity of *E. natalensis* is attributed to, among other compounds, the presence of naphthoquinones, which are the dominant secondary metabolites of the Ebenaceae family (Bryant, 1966; Van Wyk and Van Wyk,

1997). Naphthoquinones are allelopathic, antiherbivorous and endowed with antimicrobial properties against a broad spectrum of pathogens (Ioset *et al*., 1998; Sasaki *et al*., 2002).

Previous investigations on the pharmacological activity of naphthoquinones revealed their effectiveness against protozoan infections such as leishmaniasis and malaria (Hudson *et al*., 1989; Kayser *et al*., 2000). Several naphthoquinones, including 7-methyljuglone **(2)**, diospyrin **(3)**, isodiospyrin, shinanolone **(1)** and mamegakinone have already been isolated from many species of the Ebenaceae family (Van der Vijver *et al*., 1974; Ferreira *et al*., 1977; Khan, 1985). Diospyrin **(3)** and isodiospyrin, which are phytochemical constituents of *Diospyros piscatorial,* showed a wide spectrum of antibacterial activity (Adeniyi *et al*., 2000). Three naphthoquinones (diospyrin **(3)**, 7-methyljuglone **(2)** and shinanolone **(1)**) isolated from *E. natalensis* exhibited significant activity against drug-sensitive and drug-resistant strains of *Mycobacterium tuberculosis* (Lall and Meyer, 2000). Synergistic studies of these naphthoquinones with existing TB drugs and clinical trials are currently underway and therefore a substantial amount of bioactive naphthoquinones is required.

The type and level of naphthoquinones vary between different plant species and variation in bioactivity is often encountered between different parts of the same plant. In *Drosera* species, the amount of naphthoquinones varies interspecifically (Bonnet *et al*., 1984), in different tissues of the plant (Repcak *et al*., 2000) and during the growing season (Caniato *et al*., 1989). Some of these compounds are not available in synthetic form, and harvest timing is not a viable option given the spatial and temporal variation of naphthoquinone production. Sustainability can be achieved through effective breeding strategies and studies should therefore, be conducted to evaluate the effect of domestication on the concentration of secondary metabolites in medicinal plants (Appleton and Van Staden, 1995). This study was conducted to determine the presence of naphthoquinones in

seeds and variation in the content of three naphthoquinones in seedlings of *E. natalensis* grown under shadecloth.

3.3. Materials and methods

3.3.1. Germination procedure and samples

Matured seeds were collected from natural populations of *E. natalensis* at Tembe National Park, KwaZulu-Natal, RSA, in 2001. A voucher specimen (PRU: 91601) was deposited at the H.G.W.J. Schweikerdt Herbarium, University of Pretoria. Pericarps of fruits were manually removed and only naked seeds were used in the experiment. Approximately 500 seeds of *E. natalensis* were sterilized with 10% sodium hypochlorite solution for 5 minutes and then rinsed five times with autoclaved distilled water. Ten Perspex boxes were then sterilized with 100% ethanol and allowed to dry in a laminar flow. Thereafter, three layers of moist autoclaved cellulose wadding were laid in each box.

Figure 3.1. Seeds of *E. natalensis* (first stage).

Fifty seeds per box were dispersed randomly on the wet cellulose wadding and incubated in growth chambers at 30°C and exposed to a cycle of 16hr light and 8hr dark. Monitoring was conducted on a daily basis and water replenished when necessary. The seedlings were harvested at four different growth stages, using shoot and root length as variables. For the purpose of this experiment, the dormant stage, which consisted of seeds, was referred to as stage 1 (Fig. 3.1). Radicle emergence began after five weeks of incubation.

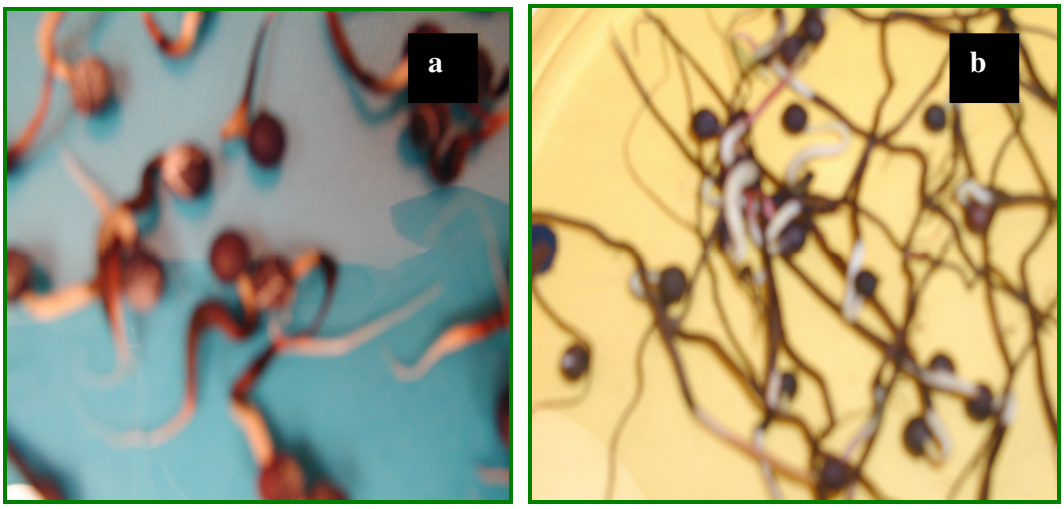


Figure 3.2. The second (a) and third (b) seedling stages of *E. natalensis*.

Three hundred seedlings were harvested at the second seedling stage (Fig. 3.2 (a)), which was identified as the point after root protrusion when the radicles were darkened in colour and were about 6 cm in length. The remaining seedlings were transplanted into individual pots containing sterile compost and transferred to a greenhouse.

The seedlings of *E. natalensis* are classified as epigeal, indicating that the cotyledons are aboveground and photosynthetic (Mayer and Poljakoff-Mayber, 1982). Consequently, hypocotyls were harvested separately from the roots, at the third stage, when they consisted of single stems with cotyledons embedded within the seed coats. The hypocotyls had an average length of 1 cm and the roots were 8 cm in length (Fig. 3.2 (b)).

Seedlings with two photosynthesizing cotyledons each characterized the fourth growth stage and like in the previous stage, the aerial parts were separated from the subterranean parts at the base of the hypocotyls.

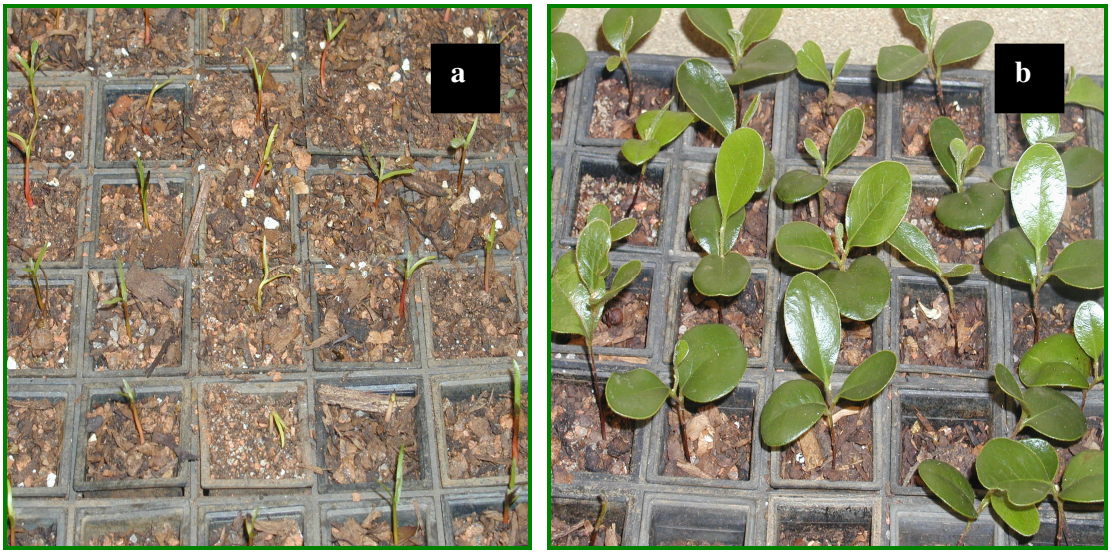


Figure 3.3. Seedlings of *E. natalensis* at fourth (a) and fifth (b) seedling stages.

The shoots were 2 cm and the roots were 10 cm in length (Fig. 3.3 (a)). The fifth growth stage consisted of seedlings with 3-5 true leaves with the length of the shoots being 3 cm whereas the roots measured 13 cm (Fig.3.3 (b)). Leaves were excised from the aerial parts at the base of their petioles. Dried seeds, shoots and roots harvested from the four seedling stages, constituted samples for analysis. All samples were air-dried at room temperature, and ground to yield homogenous powders using a Junke & Kunkel, Funkentstort KB5/10 (analysing miller).

3.3.2. Phytochemical analysis

Approximately 100 mg of ground seeds from the first stage and 100 mg of radicles from the second stage of the seedlings of *E. natalensis* were weighed out for analysis. Hundred milligrams of roots and shoots from the third, fourth and fifth seedling stages of *E.*

natalensis were also weighed out. All these samples were extracted (three times at two-hour intervals) using 2 ml of chloroform and then filtered under vacuum. The chloroform extracts were then evaporated using a nitrogen unit (Reacti-Vap, model 18780) to yield dry crude extracts. Qualitative analysis was conducted on 10 x 20 cm, TLC (Thin Layer Chromatography) plates (Merck, Silica gel $60F_{254}$) so as to determine the presence of naphthoquinones. TLC was done against authentic standards, which were isolated, purified and identified according to the published methods (Lall *et. al*., 2005; Van der Kooy *et. al*., 2006). The eluting system consisted of hexane: ethyl acetate (3:1). Separated components were visualized under visible and two ultraviolet light wavelengths (254 nm and 366 nm). Thereafter the TLC plates were then sprayed with vanillin reagent for further resolution of compounds in each sample.

All the chloroform extracts were quantitatively analyzed by means of an HPLC equipped with diode array detector UV6000LP and a Phenomenex Luna column (C18 (2) 3 µ, 150 x 4.6 mm). The mobile phase consisted of acetonitrile (MeCN): water (H_2O): acetic acid (HAc) in the ratio (62.5: 32.5: 0.5) and was used in isocratic mode at a flow rate of 0.8 mL/min at 25 ºC. The run time for each injection was 22 minutes. Each crude extract was dissolved in 2 ml acetonitrile, the sample injection volume was 10 µl and three injections per replicate were conducted. Individual naphthoquinones were identified, based on the retention time and UV spectrum of purified standards.

For quantitative analysis, pure compounds were dissolved in acetonitrile and a range of dilutions from 22.5 µg/ml to 2.25 µg/ml was prepared. The dilutions were injected into the HPLC at a volume of 10 µl, in triplicates. The absorbance wavelengths of pure 7-methyljuglone and diospyrin were 430 nm, whereas shinanolone was detected at 325 nm. Each sample extract was injected four times and their particular quantities were determined by standard curves generated for each compound. Mass of ground material (g), the standard

curves slopes, volume of injection and areas of individual peaks were used to calculate the concentration of each naphthoquinone in mg/ kg of plant material.

3.3.3. Statistical analysis

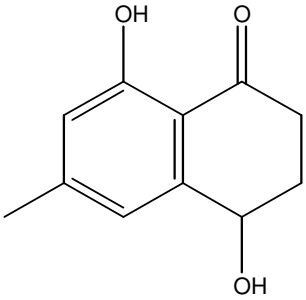
The mean values of four concentrations of each secondary compound from each sample were considered for analysis. The results were statistically analysed using one-way analysis of variance (ANOVA) and least significant differences (P = 0.05) were determined according to the MSTATC computer program.

3.4. Results and discussion

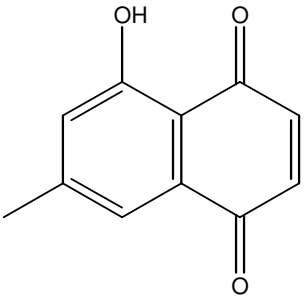
Preliminary examination of the chloroform extracts from shoots and roots of *E. natalensis* showed little variation in the chemical profiles of the respective samples analyzed (not shown). All three naphthoquinones (Fig. 3.4) were detected in all extracts except the seeds from which shinanolone **(1)** was the only compound visualized. There were very few compounds present in the fingerprints of shoot extracts and the number of compounds decreased with growth. More compounds were depicted by TLC chromatograms of root extracts. The qualitative analysis was only significant in prior assessment on the presence of the specified naphthoquinones in the crude extracts and therefore could not be used for quantification of the individual metabolites.

HPLC analysis of naphthoquinones in seed extracts revealed the presence of diospyrin **(3)**, which was not detectable from the TLC plates. Levels of the three naphthoquinones were very low or less detectible at dormancy, with shinanolone **(2)** displaying relatively higher concentrations (87.5 mg/kg) than diospyrin **(3)** and 7-methyljuglone **(2)** (6.2 and 0

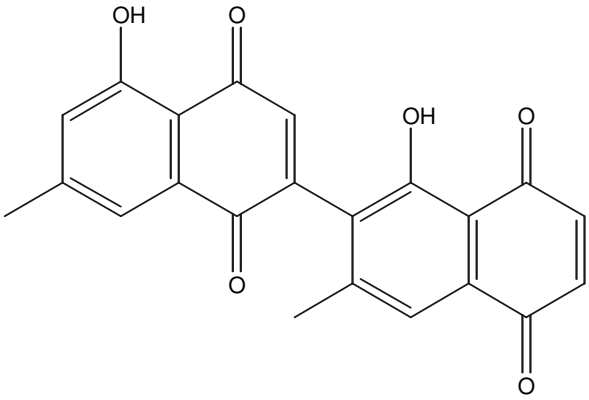
mg/kg respectively). This could be attributed to the fact that at dormancy, seeds are mainly protected from attack by microorganisms by dehydration and the impermeability of the seed coat (Baskin and Baskin, 1998; Ceballos *et al*., 1998). In general, secondary metabolites in seeds accumulate in relatively low concentrations when compared to



shinanolone **(1)**



7-methyljuglone **(2)**



diospyrin **(3)**

Figure 3.4. Naphthoquinones quantified in seeds and seedlings of *E. natalensis*.

primary products such as starch and lipids (Mayer and Poljakoff-Mayber, 1982).

The quantitative analysis of chloroform extracts from shoots clearly showed a decrease in naphthoquinone accumulation during growth (Fig. 3.5). The three naphthoquinones increased rapidly during the third growth stage, with an obviously elevated content of 7-methyljuglone **(2)** (1310 mg/kg), which was approximately three times higher than shinanolone **(1)** and diospyrin **(3)** (260.4 and 369.5 mg/kg respectively). The observed

increase correlated with the shoot development initiation of the hypocotyls, which were partly underground and therefore required the same protection as the subterranean parts. This was followed by a decline in the content of the three naphthoquinones in the fourth and fifth growth stages. There was a significant correlation ($P<0.01$) in the interaction between growth and accumulation of naphthoquinones in shoot development of *E. natalensis* seedlings.

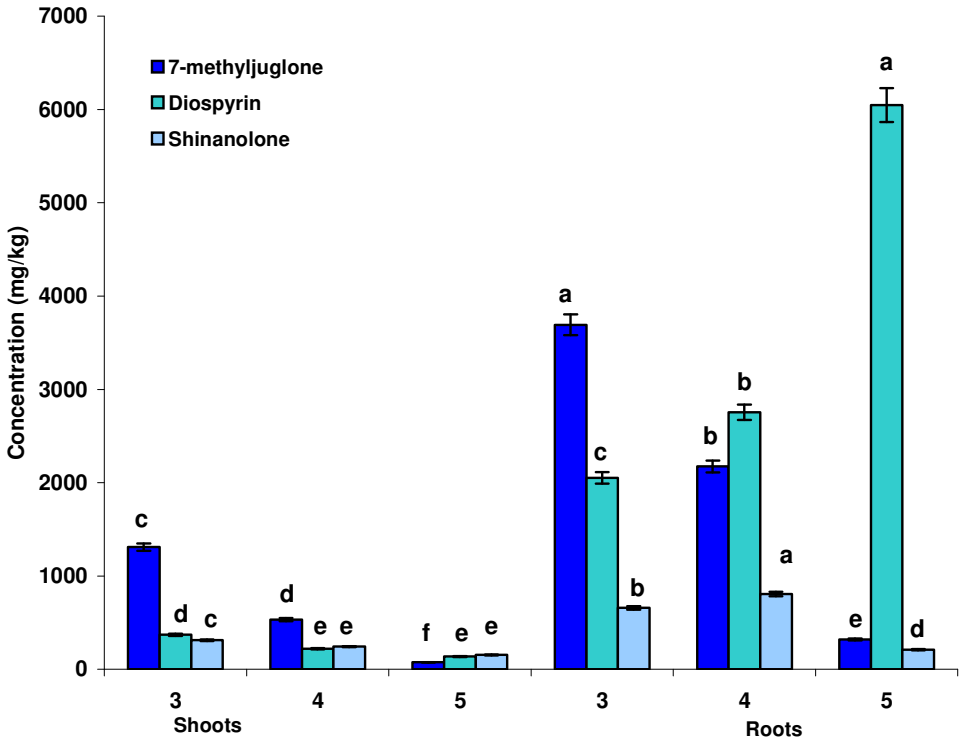


Figure. 3.5. Variation of naphthoquinones in shoots and roots at different stages of *E. natalensis* seedlings (P<0.01). Each value of a bar is a mean of four replicates. Values of the bars within each compound not followed by the same letter are significantly different.

Comparative examination of chloroform extracts from roots showed marked quantitative differences in the mean concentrations of naphthoquinones under consideration (Fig. 3.5). A significant correlation was established ($P<0.01$) between root growth and the accumulation of naphthoquinones. The concentration of shinanolone **(1)** fluctuated marginally during root growth and its levels ranged between 208.2 and 311.5 mg/kg. The accumulation of 7-methyljuglone **(2)** peaked during the third growth stage (3693 mg/kg) and decreased significantly during the fifth growth stage (319.8 mg/kg). The mean concentrations of diospyrin **(3)** showed a different pattern to those depicted by 7-methyljuglone and shinanolone, and its accumulation was directly proportional to the relative growth of the seedlings. Diospyrin **(3)** was also detected at high levels during the fifth growth stage (6048 mg/kg), when the concentrations of the other naphthoquinones were very low. This was the highest mean value quantified from the separate shoot and root samples studied.

Considering the plant as a whole, the mean concentrations of the respective naphthoquinones studied varied significantly ($P<0.01$) during the five growth stages (Fig. 3.6). All the chemical constituents considered were very low at dormancy and increased progressively up to the third stage. From this stage onward, they fluctuated independently, with the exception of diospyrin **(3)**, which accrued incrementally with growth. The content of shinanolone **(1)** ranged from 87.5 mg/kg to 1047 mg/kg and its high concentration was detected during the fourth seedling stage. 7-Methyljuglone **(2)** was quantified at a high level of 5003 mg/kg during the third seedling stage and was not detected from the seed samples. The minimum content of diospyrin **(3)** was detected initially at the first growth stage (6.2 mg/kg), whereas its highest mean levels were apparent during the fifth growth stage (6182 mg/kg). The level of consistency shown by the production of diospyrin **(3)** could also suggest its involvement in maximizing the fitness of seedlings. Seedlings are

vulnerable to pathogen attacks, and for efficient protection chemical defenses must be deployed and accumulate very early in the seedling development (Ceballos *et al*., 1998).

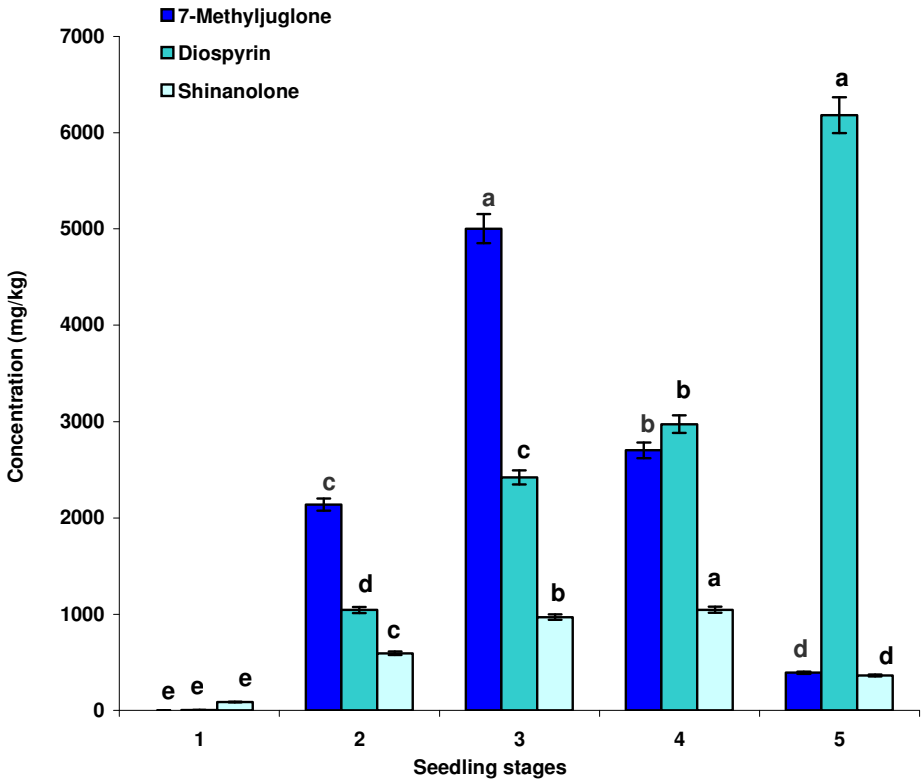


Figure 3.6. Variation of naphthoquinones in different stages of *E. natalensis* seedlings (P<0.01). Each value of a bar is a mean of four replicates. Values of the bars within each compound not followed by the same letter are significantly different.

From these results it is evident that naphthoquinones are synthesized from early stages of development. The results from this study indicate that naphthoquinones accumulated mainly in the roots, with the concentrations in shoots intermediate and those in the seeds comparatively low. Repcak *et al*. (2000) indicated that the amount of naphthoquinones vary in different tissues of the same plant, and during the growing seasons in a given

population. Based on our study, increased yields of naphthoquinones can be obtained from cultivated seedlings of *E. natalensis.* Elevated yields of shinanolone **(1)** can be obtained from the fourth seedling stage. 7-Methyljuglone **(2)** and diospyrin **(3)** could be harvested for optimum levels at the third and fifth seedling stages respectively. Large-scale production of seedlings will help in reducing the pressure that is exerted on natural population by plant gatherers.

3.5. Acknowledgements

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CHAPTER 4

Seasonal variation of naphthoquinones in *Euclea natalensis* subspecies *natalensis*

#Seasonal variation of naphthoquinones in *Euclea natalensis* subspecies *natalensis*

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4.1. Abstract

Seasonal variation of naphthoquinones was investigated from eleven plants of *Euclea natalensis* A.DC. subsp. *natalensis* (F. White) growing in natural populations of Tembe National Park, KwaZulu-Natal, South Africa. Quantitative analysis of bioactive naphthoquinones (shinanolone, 7-methyljuglone, diospyrin, isodiospyrin and neodiospyrin) present in the root extracts was conducted using High Performance Liquid Chromatography (HPLC). All these naphthoquinones have shown antituberculosis activity. The mean levels of shinanolone and 7-methyljuglone were found to be uniform in all the seasons and no statistically significant variation could be found between seasonal changes and their mean concentrations. However, the highest amount of the best antituberculosis compound, 7-methyljuglone, was found in winter in most of the plants. Accumulation of isodiospyrin and neodiospyrin varied significantly with seasonal changes ($P<0.05$). Summer and autumn appear to be appropriate seasons for detection of isodiospyrin and neodiospyrin, and the compounds were not detected in winter. A statistically significant variation ($P<0.05$) was

established between the levels of diospyrin and seasonal fluctuations. Diospyrin was detected at a mean concentration of 3.19 g/kg during spring, which was higher than the other naphthoquinones quantified in all four seasons. Hence spring seems to be the best season for the maximum availability of the second best antituberculosis compound, diospyrin. The study showed that depending on the requirement of a particular naphthoquinone, one could harvest the roots of *E. natalensis* subsp. *natalensis* in seasons recommended from this study.

Keywords: *Euclea natalensis* subspecies *natalensis*; Naphthoquinones; Shinanolone; 7-Methyljuglone; Diospyrin; Isodiospyrin; Neodiospyrin

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4.2. Introduction

Euclea natalensis subsp. *natalensis* is a perennial, woody plant species that is commonly found as a shrub, or a medium sized tree of about 2-4 m in height (Palgrave, 1991), and is native to the eastern coast of southern Africa. *E. natalensis* subsp. *natalensis* and the other subspecies of *E. natalensis* (*E. natalensis* subsp. *anguistifolia* (F. White), *E. natalensis* subsp. *magutensis* (F. White), *E. natalensis* subsp. *capensis* (F. White), *E. natalensis* subsp. *obovata* (F. White) and *E. natalensis* subsp. *rotundifolia*) (F. White) display a rather complicated ecogeographical variation and their precise delimitations may be arbitrary due to the presence of intermediates and their susceptibility to hybridization (personal

communication, Ms. Snyman, National Botanical Institute, Pretoria). These subspecies of the *Euclea* genus display an extensive flowering period ranging from May to January (Palgrave, 1991). *E. natalensis* subsp. *natalensis* is generally delineated from the other subspecies by virtue of its leaves, which are hairy, elliptic, and have pronounced wavy margins with tapering apices. Indigenous people of southern Africa harvest *Euclea* species for a number of medicinal applications. Twigs of these subspecies are locally used as toothbrushes for oral hygiene and their extracts are used for colouring purposes (Van Wyk and Van Wyk, 1997). The Zulus use its root bark to treat respiratory complications; Shangaans apply the ground powder to the skin in cases of leprosy; and the powdered plant material is also used by various ethnic groups of southern Africa to relieve headache and toothache (Bryant, 1966; Palgrave, 1991).

The bioactivity of these plant species is directly linked to the presence of naphthoquinones, which are phenolic compounds prevalent in the Ebenaceae family (Mallavadhani *et al*., 1998; Mebe *et al*., 1998). Phytochemical analysis of crude extracts from the roots of *Euclea* species revealed the presence of 7-methyljuglone and its dimers diospyrin, isodiospyrin, neodiospyrin, mamegakinone as well as shinanolone and pentacyclic triterpenoids, all of which were previously isolated (Orzalesi *et al*., 1970; Khan, 1985; Mebe *et al*., 1998). Numerous antimicrobial studies have been conducted in an attempt to evaluate the pharmacological efficacy of naphthoquinones, these including antibacterial (Adeniyi *et al*., 2000), antiprotozoal (Kayser *et al*., 2000), antifungal (Sasaki *et al*., 2002) and anti-inflammatory activities (Kuke *et al*., 1998). Owing to their cytotoxic properties, many naphthoquinones have displayed inhibitory activities on different cancer cell lines (Hazra *et al*., 2005; Wube *et al*., 2005). Various naphthoquinone derivatives are currently being synthesised in an attempt to ameliorate their cytotoxicity and to further enhance their bioavailability. Crude extracts and naphthoquinones isolated from *E.*

natalensis exhibited significant activity on a number of bacterial strains (Khan *et al*., 1978), especially against the drug-resistant strain of *Mycobacterium tuberculosis* (Lall and Meyer, 2001; Lall *et al*., 2005).

Continual harvest of plant material for both medicinal uses and ethnopharmacological studies, necessitate an understanding on how plants synthesize and accumulate bioactive compounds. Many wild species exhibit temporal and spatial variation with regard to the production of secondary metabolites. The observed variation is usually ascribed to constantly changing factors such as nutrient availability (Bi *et al*., 2005), geography (Castells *et al*., 2005), seasonal changes (Fischbach *et al*., 2002) and plant soil interactions (Wrobel *et al*., 2002). Elevated levels of toxin accumulation may possibly correlate with high pathogen attack whereas low concentrations of secondary metabolites could coincide with major phenological processes such as flowering and fruit setting. Qualitative and quantitative variation of these secondary compounds affects the curative potency of medicinal plants (McGaw *et al*., 2002). Understanding of the underlying variables that influence the synthesis and accumulation of secondary metabolites, will enhance our ability to predict their biosynthetic patterns in wild settings and to further apply strategies that will maximize yields from harvests on a sustainable basis. The main objective for this study was to establish if any significant interaction exists between seasonal changes and accumulation of bioactive naphthoquinones in a given population of *Euclea natalensis* subsp. *natalensis.*

4.3. Materials and methods

4.3.1. Experimental area

The study was conducted at the Tembe Elephant Park, KwaZulu-Natal Province, on the eastern coast of South Africa, which is located at the latitude 26º 51.56'S-27º 03.25'S, longitude 32º 24.17'E-32º 37.30'E and has an altitude of about 129 m above sea level. The climate is moist tropical/subtropical with a mean annual precipitation of approximately 721 mm and most of the rainfall is distributed between October and April. Mean annual temperature is 23.1ºC, the highest maximum temperature being 45ºC and the lowest minimum temperature recorded at 4ºC. The vegetation of Tembe Elephant Park is primarily Sand Forest occurring with other vegetation types such as woodland, thicket and wetland. The soil of the experimental site is well drained and classified as coarse sandy in texture (sand 96.1 %, silt 0.6 % and clay 1.9 %), with a pH of 5.6 and availability of P, Ca, K, Mg and Na at 8.6, 462, 88, 101 and 64 mg/kg respectively.

Figure 4.1. ***E. natalensis*** **subsp.** ***natalensis*** **growing at the Tembe Elephant Park.**

4.3.2. Plant material and extraction of compounds

Figure 4.2. Roots of *E. natalensis* subsp. *natalensis*.

Root samples were collected from eleven plants of *Euclea natalensis* subsp. *natalensis* (Fig. 4.1 and 4.2), belonging to the same population at the end of each season (30 November 2004, 28 February 2005, 31 May 2005 and 31 August 2005) for a complete annual cycle. Voucher specimens were authenticated and deposited at the H.G.W.J. Schweikerdt Herbarium, University of Pretoria (Table 4.1). The trees were tagged and further located by their GPS coordinates during each time of plant collection. The roots were ±12 mm in diameter, harvested 2 cm from the growing point and then dried at room temperature. Samples were then milled to yield homogenous powder. Three replicates of 100 mg of each ground sample were weighed and then extracted using 2 ml of chloroform,

three times and filtered under vacuum. The chloroform extracts were evaporated using a nitrogen unit (Reacti-Vap, model 18780) to yield dry crude extracts, which were then dissolved in 2 ml acetonitrile and sonicated (Elma Transsonic Waterbath) for 5 minutes.

Table 4.1. GPS points of *E. natalensis* ssp. *natalensis* plants sampled in this study.

Euclea natalensis ssp. *natalensis*	GPS location	Voucher specimen number
Plant 1	S 27° 02′ 41.7″ E 32° 25′ 18.7″	PRU095056
Plant 2	S 27° 02′ 41.7″ E 32° 25′ 18.7″	PRU095057
Plant 3	S 27° 02′ 41.7″ E 32° 25′ 18.7″	PRU095058
Plant 4	S 27° 02′ 40.8″ E 32° 25′ 19.0″	PRU095059
Plant 5	S 27° 02′ 40.8″ E 32° 25′ 19.0″	PRU095060
Plant 6	S 27° 02′ 40.7″ E 32° 25′ 18.7″	PRU095061
Plant 7	S 27° 02′ 40.5″ E 32° 25′ 18.5″	PRU095062
Plant 8	S 27° 02′ 40.3″ E 32° 25′ 19.9″	PRU095063
Plant 9	S 27° 02′ 40.7″ E 32° 25′ 20.5″	PRU095064
Plant 10	S 27° 02′ 40.7″ E 32° 25′ 20.5″	PRU095065
Plant 11	S 27° 02′ 40.8″ E 32° 25′ 19.6″	PRU095066

4.3.3. High Performance Liquid Chromatography Analysis

Chromatographic analysis was conducted on a Varian 9012 chromatograph equipped with diode array detector UV6000LP, and kept at a constant temperature of 25 ºC (Joubert *et al*., 2006). A mobile phase of MeCN: H_2O: AcOH (62.5: 32.5: 5) was used in an

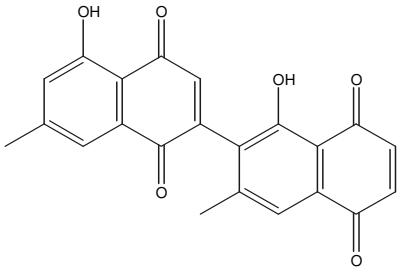
isocratic mode at a flow rate of 0.8 mL/min and the run time for each injection was 22 minutes. Standards of each compound were kindly donated by F. van der Kooy, which were used for generating calibration curves. Standards were dissolved and diluted to yield a series of concentrations in the range of 0.0022 to 0.0225 mg/ml and the wavelength used for detection was 430 nm for 7-methyljuglone, diospyrin, isodiospyrin and neodiospyrin and 352 nm for shinanolone. Ten microlitres of each solution was injected three times. Standard curves were then drawn using linear least-square regression derived from the resulting peak areas. Naphthoquinones were also identified based on the retention time of pure standards. The dissolved crude extracts were then injected three times per replicate.

4.3.4. Statistical analysis

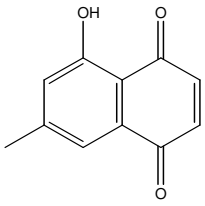
Mean values of three concentrations of each secondary compound from each crude extract were considered for analysis. A one-way ANOVA was applied to the acquired analytical data to determine the statistical significance of variation in the content of naphthoquinones with seasonal changes. This was based on the assumption that the differences among the means during different seasons were significant at $P<0.05$.

4.4. Results and Discussion

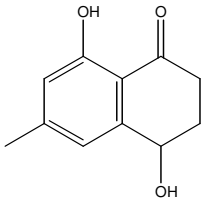
The relative levels of the five naphthoquinones (see Fig. 4.3) differed distinctly within the subspecies throughout the experimental period. Variations in the levels of naphthoquinones among members of *E. natalensis* subsp. *natalensis* were substantial, ranging from zero to more than five times that of their mean values. Individuals of a population may display variation in secondary metabolites on a small spatial scale. Zenk *et*



diospyrin



7-methyljuglone



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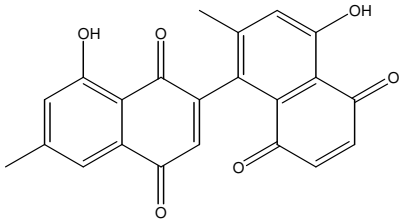
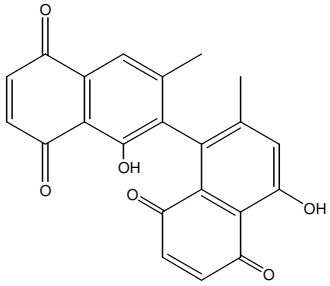


neodiospyrin



isodiospyrin

Figure 4.3. Naphthoquinones isolated from *E. natalensis* subsp. *natalensis*

al. (1969) documented empirical evidence of intraspecific variation of naphthoquinones from members of the *Drosera* populations. A significant qualitative variation was also encountered in different seasons and between several members of a population harvested at the same time. Some of the HPLC chromatograms revealed qualitatively similar profiles although with highly variable levels of these compounds. The reason for such differences in accumulation of naphthoquinones in *E. natalensis* subsp. *natalensis* does not seem to be

due to the effect of environmental conditions, but rather the age of the sampled plant species, in view of the fact that it was the only variable that we could not verify.

The concentration of shinanolone and 7-methyljuglone was found to be uniform in all the seasons and no statistical correlation could be established between seasonal changes and their mean concentrations at the population level. Seasonal mean values of the two monomeric naphthoquinones varied by 2.8- and 3.1-fold for shinanolone and 7-methyljuglone respectively. Despite the close mean values recorded for both monomeric compounds and the apparent uniformity reflected by their profiles (Figure 4.4), there were many instances whereby higher levels were detected in one plant as compared to the other plants, in a given season. The concentration of shinanolone varied from 0 mg/kg to 0.772 g/kg and the concentration of 7-methyljuglone ranged from 0 g/kg to 3.77 g/kg between individuals of a population and among seasons. The observed high variability from members of *E. natalensis* subsp. *natalensis* is consistent with the results from Kämäräinen *et al*. (2003) who reported on the disparate regional and habitat differences in the production of 7-methyljuglone in clones of *Drosera rotundifolia* populations. Peak levels of shinanolone and 7-methyljuglone were found in summer and spring correspondingly.

The accumulation of isodiospyrin and neodiospyrin were less pronounced in some individual plant species of *E. natalensis* subsp. *natalensis*. A marginally significant variation ($P<0.05$) was established in the production of isodiospyrin and neodiospyrin and with seasonal changes. The seasonal mean concentrations of these secondary metabolites ranged from 0 to 0.35 g/kg for isodiospyrin and 0 to 0.087 g/kg for neodiospyrin during the period of study. Concentrations of the respective dimeric naphthoquinones differed significantly between individuals of the population harvested during the same season. This lack of a definite pattern in the levels of isodiospyrin and neodiospyrin observed within species, could be attributable to the age factor of the plant species. Bazzaz *et al*. (1987)

pointed out that variation in the synthesis of secondary metabolites could be encountered in relation to age among members of the same plant species. The maximum levels of isodiospyrin and neodiospyrin recorded per individual plant were 1.72 g/kg during summer and 0.488 g/kg in autumn respectively. Neodiospyrin could not be detected from all the samples analysed during winter and summer, whereas isodiospyrin did not integrate in any of the chromatographic profiles evaluated from winter samples.

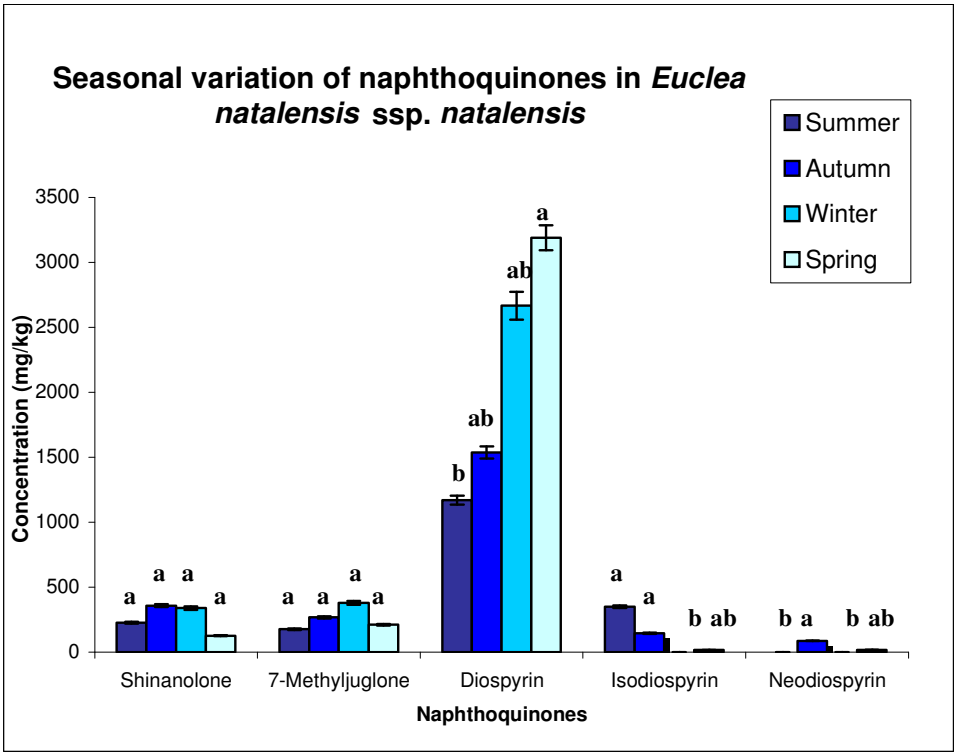


Figure 4.4. Seasonal variation of naphthoquinones in *E. natalensis* ssp. *natalensis*. Each bar represents a mean of 11 values.

Diospyrin was the only naphthoquinone that was detected in all the samples that were considered throughout the experimentation, although at variable amounts. The evident qualitative uniformity revealed by the chromatograms of diospyrin, could possibly be linked to its biosynthetic pathway and ecological role. There appear to be competition for common substrates, presumably the monomeric compounds, between the three dimeric

naphthoquinones with the diospyrin pathway being the most favourable, thus accumulating in relatively high concentrations. This could suggest its ecological significance in enhancing the fitness cost of *E. natalensis* subsp. *natalensis* plants, by conferring resistance against pathogens in their rhizosphere. Although the ecological role of these naphthoquinones in *E. natalensis* subsp. *natalensis* is unknown, secondary metabolites are only produced in large quantities when they are needed, at a particular stage in the life cycle, at certain seasons and in those tissues that require most protection (Salminen *et al.*, 2001; Fischbach *et al.*, 2002). Mean value concentrations of diospyrin exhibited a significant variation with seasonal changes ($P<0.05$), and varied from 1.17 g/kg to 3.19 g/kg over the annual cycle. Spring appeared to be the favourable season when high levels of diospyrin are present in most individuals of the population under investigation. At the subspecies level, the quantity of diospyrin differed significantly among members whereby minimum and maximum concentrations detected were 0.199 g/kg and 6.79 g/kg respectively, which is approximately 34.1-fold increase than that of the lowest amount quantified for this compound.

The observed lack of correspondence and synchrony in the synthesis and accumulation of naphthoquinones between individuals of a *E. natalensis* subsp. *natalensis* population could be best explained in terms of plant age, rather than the availability of resources or other varying environmental conditions. Variable responses in the biosynthesis of naphthoquinones in *E. natalensis* subsp. *natalensis* could suggest its dependence on the genotype. It should be noted that the specific ages of the individual plants were unknown to us and differential physiological responses may result in unpredictable responses of compounds with rapid turnover. The resultant disparate expression of the biosynthesis of naphthoquinones may be ascribed to the different stages of ontogenic shifts and phenological development found in these plant species. Laitinen *et al.* (2005) found large

infraspecific variation in secondary chemistry, which was attributed to the genotype and ontogeny of the members of a *Betula pendula* population. The observed variation in the accumulation of naphthoquinones shows that high yields of the desired compounds can be harvested during the specified seasons from a population of *E. natalensis* subsp. *natalensis*.

4.5. Acknowledgements

We would like to thank National Research Foundation and Innovation Fund for their financial assistance. Mahdi Ziaratnia is acknowledged for his help with statistical interpretation.

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CHAPTER 5

Fertilization-induced changes in growth parameters and antimycobacterial activity of *Euclea natalensis*

Fertilization-Induced Changes in Growth Parameters and Antimycobacterial Activity of *Euclea natalensis* (Ebenaceae)

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5.1. ABSTRACT

The effect of Nitrogen: Phosphorus: Potassium (NPK) (2:1:2 (44)) fertilizers on growth, accumulation of bioactive naphthoquinones (shinanolone, 7-methyljuglone, diospyrin, isodiospyrin and neodiospyrin) and antimycobacterial activity was investigated in seedlings of *Euclea natalensis* (Ebenaceae) grown in shadehouse and in field conditions. Seedlings were subjected to three differential fertilization regimes (Treatment 1 at 40 g/l, Treatment 2 at 20 g/l and Treatment 3 at 10 g/l). Treatment 2 enhanced the vegetative performance of seedlings grown under shadehouse conditions. A significant correlation was established between the concentration of shinanolone ($P<0.01$), isodiospyrin ($P<0.05$) and neodiospyrin ($P<0.05$) with field-grown seedlings. Application of NPK fertilizers

significantly (P<0.05) increased the accumulation of neodiospyrin in seedlings subjected to shadehouse conditions. The most potent naphthoquinone, 7-methyljuglone was found to be abundant in all the extracts and was quantified at high mean concentration of 10.2 g/kg from shade-grown seedlings. Extracts acquired from field-grown seedlings were more active against *Mycobacterium smegmatis* with a lowest MIC value of 0.78 mg/ml. Extracts from the control group and Treatment 1 (10 g/l), which had less applied fertilizers, were more active against strains of *M. tuberculosis* with MIC value of 10 µg/ml. Our study indicated that subjecting seedlings to homogenous environments coupled with higher levels of fertilizers could have a negative impact on the antimycobacterial activity of *E. natalensis*.

Keywords: *Euclea natalensis*; NPK fertilizers; field; shadecloth; naphthoquinones; antimycobacterial activity

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5.2. INTRODUCTION

The recent resurgence of interests in medicinal plants and plant-derived secondary metabolites is primarily aimed at discovering novel secondary metabolites, which can counteract resistance from pathogens and circumvent side effects that are typically associated with most indispensable therapeutic drugs. Plant material harvested from wild stocks cannot meet the escalating demand of natural products in the growing global market of medicinal and aromatic plants (Nigro, 2004). It has become inevitable to guarantee a sustained supply of renewable resources of raw plant materials to both local and global markets by optimization through effective propagation and breeding methods (Jäger and

Van Staden, 2000). Introduction of medicinal plants into cultivation will not only ensure a continued supply of phytochemicals, but will also ascertain conservation of genetic resources from wild populations. This will alleviate their over-exploitation and relieve the pressure exerted on populations of wild plant species.

Generally, plants synthesize and accumulate secondary metabolites in response to the pressure exerted by biotic and abiotic ecological factors found within their immediate environment. If such interactions are broken, then these plants species are most likely to yield trace amounts of the compounds of interest. Empirical studies have revealed that domestication of wild species, which involves optimization of growth conditions, may affect their allocation patterns resulting in trade-offs between agronomical traits and chemical qualities (Máthé, 1988). Cultivation trials necessitate parallel studies, which are mainly aimed at elucidating factors such as exogenous application of elicitors, fertilizers and soil properties that have been shown to affect plant productivity and quality. The medicinal attributes of each harvest need to be systematically evaluated, using fingerprinting techniques and antimicrobial assays to assure standardization and safety of extracted phytochemicals (McGaw *et al*., 2002)

Euclea natalensis A.DC., a member of the Ebenaceae family, is endowed with a broad variety of medicinal and horticultural qualities. Many *Euclea* species are frequently gathered by indigenous people of southern Africa to treat respiratory complications such as chest pains, bronchitis, pleurisy and asthma (Bryant, 1966). Its ground powder is topically applied to the skin in cases of leprosy and used by some ethnic groups to relieve toothache and headache (Palgrave, 1991). During pharmacological evaluation of crude extracts and naphthoquinones (shinanolone, 7-methyljuglone, diospyrin, isodiospyrin and neodiospyrin) isolated from *E. natalensis*, it was found that these compounds have antibacterial activity against drug-resistant and drug-sensitive strains of *Mycobacterium tuberculosis* (Lall and

Meyer, 2001; Lall *et al*., 2005). Some of these bioactive naphthoquinones accumulate in minute amounts, and are not available in synthetic form. As a result, more plant material is required for further studies such as pre-clinical and clinical trials. This prompted us to conduct a study in an attempt to assess the effect of fertilizers on the synthesis and accumulation of these antitubercular compounds in seedlings of *E. natalensis*, cultivated in field setting and under shadecloth conditions. The bioactivity of crude extracts from the seedlings of *E. natalensis* was evaluated against strains of *M. smegmatis* and *M. tuberculosis*.

5.3. MATERIALS AND METHODS

5.3.1. Study area

Cultivation trials were conducted at the Experimental Farm of the University of Pretoria, South Africa, during November 2003-August 2005. The soil of the experimental site is well drained and sandy loam in texture (sand 62.9 %, silt 22.9 % and clay 7.1 %), with a pH of 7.6 and available P, Ca, K, Mg and Na at 102.9, 2854.7, 258.7, 397.3 and 49 mg/kg respectively.

5.3.2. Plant material

Seeds of *Euclea natalensis* were collected from their natural populations in Tembe Elephant Park, KwaZulu-Natal, South Africa in 2002. A voucher specimen (PRU91601) was authenticated and deposited at the H.G.W.J. Schweikerdt Herbarium, University of Pretoria. The seeds were germinated in growth chambers set at 30 ºC and exposed to a

cycle of 16 h dark and 8 h light. After radicle emergence, seedlings were transplanted into pots with a soil mixture of river sand: topsoil: compost (bark) (1:1:2) and then kept in a shadehouse while waiting for the 3-4 true leaf stage. After 10 months of establishment the seedlings were divided into two groups, one of which remained under the shadehouse, while the other group was transplanted into the field (Fig. 5.1). The experimental layout was a randomised block design and the seedlings were planted at a space of 30 cm apart in the field.

Figure 5.1. Seedling of *Euclea natalensis* grown under shadecloth (a) and field (b) conditions

Each group was further subdivided into four subgroups, which were then subjected to four respective treatments. Water-soluble foliar feed NPK (2:1:2) was applied at three different concentrations. Treatments tested were Treatment 1 (40 g/l), Treatment 2 (20 g/l) (suppliers' recommended concentration) and Treatment 3 (10 g/l). The control group received only supplemental water. Treatments were applied at 200 ml per plant once in every two weeks for one year. Treatments were the same for both shadehouse and field

seedlings. Irrigation was carried out three times per week in the morning (8 am). The first harvest was conducted after 6 months of application of fertilizers. The second plant collection was done after 12 months of treatment. Leaves were excised from the aerial parts. Heights, and fresh and dry weight of the seedlings were measured for their vegetative performance. Roots of seedlings were dried at room temperature and then ground to a fine powder for further analysis.

5.3.3. Extraction

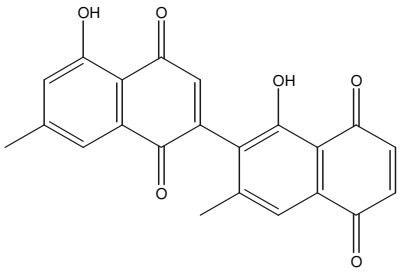
Three replicates of 100 mg of each powder were weighed and then extracted three times, for 48 h using 500 ml of chloroform, and filtered under vacuum. The filtrates were evaporated using nitrogen unit (Reacti-Vap, model 18780) to yield dry crude extracts.

5.3.4. Phytochemical analysis

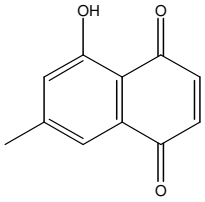
Part of the crude extracts was dissolved in acetonitrile (2 ml) and sonicated (Elma Transsonic Waterbath) for 5 minutes. Dissolved extracts were centrifuged (microcentrifuge Millipore) and subjected to quantitative analysis using High Performance Liquid Chromatography (HPLC). Chromatographic analysis was carried out using a Varian 9012 chromatograph equipped with a diode array detector UV6000LP, and kept at a constant temperature of 25 ºC (Joubert *et al*., 2006). A mobile phase of acetonitrile (MeCN): water (H_2O): acetic acid (AcOH) (62.5: 32.5: 5) was used in an isocratic mode at a flow rate of 0.8 ml/min and the run time for each injection was 22 minutes. Each crude extract (0.1 mg) was dissolved in 2 ml acetonitrile, the sample injection volume was 10 µl and three replicates were injected.

Naphthoquinones (Fig. 5.2) were identified based on their retention times and UV-VIS spectra compared to that of purified standards (Joubert *et al*., 2006). For quantification of

the naphthoquinones present in the samples, each pure compound was dissolved and diluted to yield a series of concentration in the range of 2.25 µg/ml to 22.5 µg/ml, and three injections of 10 µl were performed for each dilution. The wavelength used for detection was 430 nm for 7-methyljuglone, diospyrin, isodiospyrin and neodiospyrin whereas shinanolone was detected at 325 nm. Calibration curves were generated for each compound using linear least-square regression equations derived from the resulting peak areas.



diospyrin



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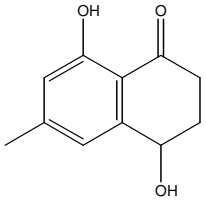
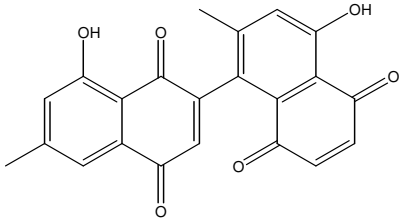
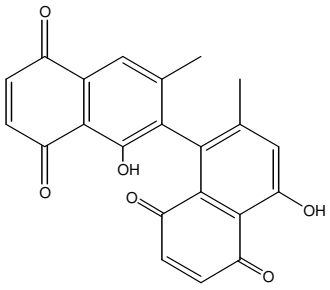


shinanolone



neodiospyrin



isodiospyrin

Figure 5.2. Naphthoquinones quantified in seedlings of *Euclea natalensis*

5.3.5. *Mycobacterium* species

The microorganisms *Mycobacterium smegmatis* (MC^2 155) and *M. tuberculosis*, H37Rv (ATCC 27264) were obtained from American Type, MD, USA Culture Collection. Mycobacterial strain of *M. smegmatis* was cultured and maintained on Middlebrook 7H11 agar base (Sigma-Aldrich Chemical Co., South Africa) for 24h at 37 ºC. *M. tuberculosis* was plated onto Löwenstein-Jensen medium and allowed to grow for 3-4 weeks at 37ºC.

5.3.6. Microdilution screening assay using *M. smegmatis*

M. smegmatis colonies were transferred from the surface of the agar base into Middlebrook 7H9 broth base (Sigma-Aldrich Chemical Co., South Africa), which was supplemented with Oleic-Albumin Dextrose-Catalase (OADC). The suspension was homogenized with a vortex mixer (Heidolph, Germany) and allowed to settle for 30 minutes. The supernatant was collected and adjusted with Middlebrook 7H9 broth base to an optical density of 0.2 (log-phase) at λ 550 nm (Beckman DU-720 UV spectrophotometer). The bacterial concentration of the suspension was 1.26 x 10^8 colony-forming units per millilitre (CFU ml^{-1}) at the beginning of the test (Mativandlela *et al.,* 2006).

The crude extracts were each prepared by first dissolving in 100 % Dimethyl Sulfoxide (DMSO) and then adjusted to 10 % DMSO with Middlebrook 7H9 broth base to yield stock solutions with a concentration of 100 mg/ml. Growth control experiments on *M. smegmatis* indicated that a concentration of 2.5 % DMSO/well or less did not inhibit the bacterial growth. The microdilution assay was conducted in 96-well microtitre plates using Middlebrook 7H9 broth base as a blank at 100 µl/well (Newton *et al.,* 2002). For the test, 100 µl of each 10 % DMSO, stock solutions from crude extracts, ciprofloxacin and broth base were added to the first rows of the plates. Ciprofloxacin served as the positive control drug with a concentration range of 0.31 to 9.76 mg/ml (Salie *et al.,* 1996). 10 % DMSO and the culture medium were used as the solvent control and growth control respectively.

The experiment was done in triplicate. Serial two-fold dilutions of each sample to be tested were prepared with Middlebrook 7H9 broth base to yield volumes of 100 µl/well with final concentrations of extracts ranging from 0.39 to 25 mg/ml. The bacterial suspension was added to each well at 100 µl to give a final volume of 200 µl/well and the plates were incubated at 37 ºC for 24 hr.

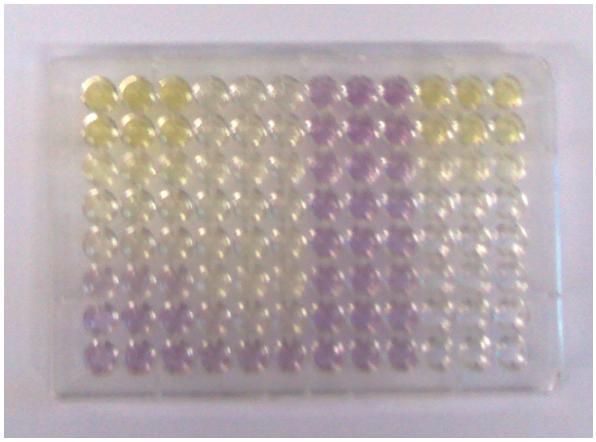


Figure 5.3. Microtitre plate used in the microdilution assay

The minimum inhibitory concentration (MIC) of each extract was determined by adding 40 µl of 0.2 mg/ml of *p*-iodonitrotetrazolium chloride (INT, Sigma-Aldrich Chemical Co., South Africa) and incubated at 37 ºC for 1 hr (Eloff, 1998). Viable bacteria reduced the yellow dye to a pink colour (Fig. 5.3). MIC was defined as the lowest sample concentration that prevented this chemical reduction and displayed absolute inhibition of the bacterial growth. The minimum bactericidal concentration (MBC) was determined by adding 50 µl of aliquots from wells, which did not show any growth after incubation during the MIC assay, to 150 µl of broth. These were incubated for 48 hrs. The viability of *M. smegmatis* was tested as in the MIC assay. MBC was regarded as the lowest concentration of extract, which did not prevent bacterial growth after 48h of incubation.

5.3.7. Antitubercular rapid radiometric assay using *M. tuberculosis*

The antitubercular activity of the crude extracts obtained from the first and the second harvest of *E. natalensis* seedlings was conducted using the BACTEC (Becton Dickinson Diagnostic Instrument, Sparks, md) (Fig. 5.4) radiometric respiratory technique as previously prescribed (Lall and Meyer, 2001; Mativandlela *et al.,* 2006). Crude extracts were dissolved in DMSO to yield stock solutions of 5 mg/ml. Control experiments showed that a final concentration of 1% DMSO in the medium had no adverse effect on the growth of *M. tuberculosis*. All the extracts were tested at concentrations ranging from 5 to 25 µg/ml based on their respective MIC values on *M. smegmatis.* Three concentrations were assayed in duplicates for each test sample. The primary drug isoniazid (INH) (Sigma-Aldrich, South Africa) was used as a drug control at a final concentration of 0.2 µg/ml.

Figure 5.4. BACTEC TB-460 instrument

A homogenous culture (0.1 ml of *M. tuberculosis*, yielding 1 x 10^4 to 1 x 10^5 CFU/ml) was inoculated into the vials containing extracts as well as those of the controls. Two growth control groups were assayed in triplicates. The initial inoculum and 1% DMSO

were added to the first growth control replicates. In the second growth control group, each vial was inoculated with 100 µl of 1:100 dilution of the inoculum to produce an initial concentration representing 1% of the bacterial population (1 x 10^2 to 1 x 10^3 CFU/ml). The MIC was defined as the lowest concentration of the extract that inhibited >99% of the bacterial population. *Mycobacterium* growing in 7H12 medium containing ^{14}C-labelled substrate (palmitic acid) uses the substrate and produces $^{14}CO_2$. The amount of $^{14}CO_2$ detected is expressed in terms of the growth index (GI) (Middlebrook *et al.*, 1977). Inoculated bottles were incubated at 37 ºC and each bottle was assayed daily to measure GI, until cumulative results were interpretable (Lall and Meyer, 2001).

5.3.8. Statistical analysis

Statistical analyses were performed by means of the SAS (SAS Institute, Inc, Cary, NC, Version 7.01, USA) Programme. The mean values obtained from different groups were compared in a one-way ANOVA, assuming that the differences between the means were significant at $P<0.05$, $P<0.01$ and $P<0.001$.

5.4. RESULTS

5.4.1. Vegetative performance

Vegetative performance of *E. natalensis* seedlings in relation to fertilizer treatments during the growing season of November 2003 and August 2005 is shown on Table 5.1. Growth parameters differed between the two groups and between treatments, with seedlings from the shadehouse showing more plant vigour than the field-grown plants. No significant correlation could be established between treatment and the measured growth factors from field-grown seedlings. Mean values of seedlings from the shadehouse during the second harvest were two-fold or more than those harvested from the field. A significant variation ($P<0.001$) was observed between treatment and the growth factors of shadehouse seedlings.

Treatment 2, which is the recommended dosage, enhanced vegetative performance with the mean values of fresh weight of shoots and roots being twice as much as their respective control mean values. A significant variation ($P<0.001$) was also found between harvest and treatment from shadehouse seedlings, as more biomass was collected from the second harvest. Seedlings harvested from the shadehouse under Treatment 2 had higher mean values for all the vegetative factors measured and differed considerably from the field-grown seedlings that were subjected to the same treatment.

5.4.2. Extraction yields

Table 5.2 shows the comparative analysis of the crude extracts obtained from the first and the second harvest in seedlings of *E. natalensis* grown under field and shadehouse conditions. Seedlings harvested from the control group under field conditions yielded the highest percentage of crude extract per plant material (6.5 %) in all the groups. The

Table 5.1. Mean values[a] of growth parameters of *Euclea natalensis* seedlings grown under field and shadehouse conditions

		Field seedlings					Shadehouse seedlings				
Harvest	Treatment	Height (cm)	FWS (g)	FWR (g)	DWS (g)	DWR (g)	Height (cm)	FWS (g)	FWR (g)	DWS (g)	DWR (g)
First											
	Control	30.0±4.4	0.8±0.4	4.3±1.8	0.7±0.3	3.1±1.6	30.1±4.7	2.9±1.6	15.9±3.5	1.6±0.9	9.8±2.8
	Treatment 1	32.3±4.7	1.5±0.1	8.7±1.9	1.3±0.1	6.1±1.5	30.1±6.4	1.6±0.6	15.8±0.7	1.8±0.2	9.0±2.0
	Treatment 2	25.3±3.5	2.2±0.5	7.3±5.6	1.4±1.2	4.9±3.4	36.0±1.0	5.3±0.6	21.4±8.3	2.6±0.3	9.7±3.8
	Treatment 3	28.0±3.6	1.4±1.0	6.3±0.7	1.3±0.9	4.3±0.4	34.7±7.5	5.8±1.9	19.5±6.6	2.9±0.9	6.8±5.6
Second											
	Control	39.0±4.0	3.7±1.5	9.6±4.4	2.2±0.9	6.2±2.9	93.3±6.7	4.1±2.5	20.9±3.4	2.1±0.3	13.5±3.3
	Treatment 1	48.7±3.1	3.3±0.3	10.4±1.3	1.9±0.2	6.2±2.2	66.0±6.2	4.8±1.1	19.5±3.3	2.1±0.4	15.6±2.0
	Treatment 2	49.0±6.6	2.4±2.0	8.7±2.2	1.8±0.3	5.9±1.1	120.0±8.0	6.3±1.3	32.1±15.2	3.7±0.8	20.1±1.8
	Treatment 3	46.0±9.0	2.6±0.9	12.1±1.6	1.6±0.5	7.7±1.5	94.0±7.0	6.8±1.4	30.4±11.2	3.6±1.1	23.9±2.9
Significance[b] due to:											
Harvest		***	**	*	*	*	***	*	*	*	***
Treatment		NS	NS	NS	NS	NS	***	**	*	**	**
Harvest X Treatment		NS	NS	NS	NS	NS	***	NS	*	NS	NS

[a]Each mean value is an average of three replications ± standard deviation. [b]By analysis of variance: ***, P < 0.001; **, P < 0.01; *, P < 0.05; NS, not significant

FWS = Fresh weight of shoots, FWR = Fresh weight of roots, DWS = Dry weight of shoots, DWR = Dry weight of roots.

percentages of crude extract per plant material obtained from the field-cultivated seedlings were always higher than their corresponding treatments from the shadehouse seedlings.

Table 5.2. Total yields of naphthoquinones from seedlings of *Euclea natalensis*

Harvest	Treatment	Field Mass in (g) and in (%) (in parenthesis)	Shadehouse Mass in (g) and in (%) (in parenthesis)
First			
	Control	0.35 (2.1)	0.24 (0.5)
	Treatment 1	0.47 (2.9)	0.53 (2.1)
	Treatment 2	0.50 (3.9)	0.48 (1.9)
	Treatment 3	0.41 (3.8)	0.54 (2.0)
Second			
	Control	1.17 (6.5)	0.55 (2.3)
	Treatment 1	0.63 (3.9)	0.21 (2.1)
	Treatment 2	0.62 (4.0)	1.01 (2.3)
	Treatment 3	0.67 (4.1)	1.07 (3.4)

5.4.3. Phytochemical analysis

Table 5.3 shows the comparative analysis of the effect of NPK fertilizers on the concentration of active constituents of *E. natalensis*. A positive interaction was established between the concentration of shinanolone ($P<0.01$), isodiospyrin ($P<0.01$) and neodiospyrin ($P<0.05$) with fertilization treatments in field-grown seedlings. Shinanolone and neodiospyrin were quantified at high mean values of 4.5 g/kg and 3.2 g/kg respectively, from seedlings subjected to Treatment 1 under field conditions during the second harvest. Treatment 2 enhanced the accumulation of isodiospyrin for both groups.

Table 5.3. The concentration[a] of naphthoquinones in field-grown and shadehouse seedlings of *Euclea natalensis*

		Field seedlings					Shadehouse seedlings				
Harvest	Treatment	Shinanolone (g/kg)	7-Methyljuglone (g/kg)	Diospyrin (g/kg)	Isodiospyrin (g/kg)	Neodiospyrin (g/kg)	Shinanolone (g/kg)	7-Methyljuglone (g/kg)	Diospyrin (g/kg)	Isodiospyrin (g/kg)	Neodiospyrin (g/kg)
First											
	Control	2.1±2.2	6.4±4.2	3.7±1.7	0.24±0.2	2.4±2.8	2.6±2.7	4.6±4.9	4.4±3.1	0.8±0.8	1.1±1.2
	Treatment 1	3.2±3.3	6.6±7.1	2.5±1.8	0.23±0.6	3.2±3.6	1.5±2.1	3.4±3.9	6.8±5.7	0.8±0.8	0.7±0.9
	Treatment 2	2.7±2.9	3.6±3.8	2.8±1.8	0.61±0.5	2.5±2.8	3.2±3.4	2.0±3.1	5.7±4.2	0.7±1.0	1.4±1.9
	Treatment 3	1.8±1.9	3.6±4.2	2.3±1.3	0.34±0.2	1.6±2.0	3.4±3.5	2.7±2.8	4.1±3.2	0.7±0.4	1.4±1.8
Second											
	Control	4.1±2.3	6.9±4.9	3.0±2.3	0.3±0.2	1.2±1.5	3.3±3.7	10.2±11.1	5.3±3.9	0.8±0.7	0.9±1.2
	Treatment 1	4.5±4.6	6.5±7.1	3.6±2.2	0.4±0.3	1.7±2.1	3.7±4.1	4.8±5.7	5.9±3.9	1.2±1.1	0.4±4.2
	Treatment 2	3.8±4.0	5.6±6.0	3.7±4.6	0.8±1.3	1.8±1.9	4.9±4.2	8.2±9.1	5.4±4.7	0.6±0.6	2.1±2.8
	Treatment 3	2.5±2.3	4.1±4.4	2.5±0.9	0.4±0.2	1.0±1.1	4.1±4.2	6.3±6.9	5.9±5.6	1.0±1.1	0.5±0.6
Significance[b] due to:											
Harvest		NS	NS	NS	NS	**	**	***	NS	NS	NS
Treatment		**	*	NS	**	*	NS	**	NS	NS	*
Harvest X Treatment		NS	NS	NS	NS	NS	NS	*	NS	NS	*

[a]Each concentration represents a mean value of three replications ± standard deviation. [b]By analysis of variance: ***, P < 0.001; **, P < 0.01; *, P < 0.05; NS, not significant

There was no significant difference detected from the respective mean values of 7-methyljuglone and diospyrin with treatments from field-cultivated seedlings, although their accumulation increased with time. A significant increase ($P<0.05$) in the concentration of neodiospyrin was observed with seedlings from shadehouse treatment.

A negative correlation ($P<0.001$) was found between the accumulation of 7-methyljuglone and treatment under shadehouse conditions. It is worth noting that 7-methyljuglone; the most abundant and potent (Lall *et al*., 2005) naphthoquinone was quantified at a highest mean value (10.2 g/kg) from samples acquired from control groups under shadehouse conditions during the second harvest. The second harvest yielded a relatively high concentration of naphthoquinones except for neodiospyrin, in which the first harvest was mostly two-fold the amount acquired from the second harvest.

5.4.4. Antimycobacterial activity of *E. natalensis* seedlings

The antimycobacterial activity of root extracts from seedlings of *E. natalensis* obtained during the first and the second harvests, is illustrated in Table 5.4. Root extracts of *E. natalensis* seedlings grown under field conditions were generally more active against the bacterial strain of *M. smegmatis* as compared to extracts acquired from roots of seedlings maintained under shadehouse setting. The lowest MIC of 0.781 mg/ml was obtained from the control and Treatment 1 seedlings, which were collected during the second harvest under field conditions. These extracts had high levels of 7-methyljuglone, which is known to be highly active (Lall *et al*., 2005). A relatively higher MIC of 3.13 mg/ml was found from field-grown seedlings subjected to Treatment 3 from the second harvest. The bioactivity of root extracts acquired from shadehouse seedlings during the second harvest was comparatively lower than those of field plants and the MIC ranged

Table 5.4. Antimycobacterial activity of *Euclea natalensis* seedlings

		M. smegmatis				*M. tuberculosis*			
		First harvest		Second harvest		First harvest		Second harvest	
Seedlings Field	Treatment	[a]MIC (mg/ml)	[b]MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (µg/ml)	ΔGI[c]	MIC (µg/ml)	ΔGI[b]
	Control	6.25	25	0.78	12.5	20	0.0±0.0	10	9.0±0.0
	Treatment 1	6.25	NA*	0.78	12.5	20	23±4.24	10	0.5±0.5
	Treatment 2	6.25	25	1.56	25	25	9.5±0.71	15	1.5±3.5
	Treatment 3	6.25	25	3.13	25	25	7±4.24	15	0.5±0.7
Shadehouse									
	Control	12.5	NA*	3.13	25	20	1±0	10	-2.0±0.0
	Treatment 1	6.25	25	1.56	12.5	25	10.5±0.71	15	0.0±0.0
	Treatment 2	25	NA*	6.25	25	25	6±1.41	15	1.5±0.7
	Treatment 3	6.25	25	3.13	25	25	14.5±4.95	15	0.5±0.7
RA		0.61	1.22	0.61	1.22	0.12	-6.50±0.72	0.12	-6.50±0.72

[a]Minimum inhibitory concentration, [b]Minimum bactericidal concentration, [c]ΔGI value (mean ± standard deviation) of the control vial was 29,5±0.71 for the sensitive strain. NA* ; no activity, RA: reference antibiotics, ciprofloxacin and isoniazid for *M. smegmatis* and *M. tuberculosis* respectively.

from 1.56 to 6.25 mg/ml. MBC values for both groups during the second harvest were higher and recorded between 12.5 and 25 mg/ml. The second harvest yielded more active extracts than the first harvest, probably due to the increase of bioactive compounds (Table 5.4). The MIC values of the crude extracts during the first harvest were higher and ranged from 6.25 to 25 mg/ml. Their respective MBC values were even higher and in some instances there was no activity at all.

Field-grown seedlings showed a higher antitubercular activity than the seedlings grown under shadehouse, as was the case in the *M. smegmatis* experiment (Table 5.4). There appears to be a high level of selectivity of *E. natalensis* activity towards *M. tuberculosis* as the extracts were active at lower concentrations. Extracts from the control group and Treatment 1, which had less fertilizer applications were more active against strains of *M. tuberculosis* with MIC value of 10 µg/ml. The shadehouse conditions did not enhance the antimycobacterial activity of the root extract.

5.5. DISCUSSION

Biomass production increased significantly as a result of fertilizer applications with seedlings from the shadehouse displaying improved vegetative performance when compared to the field- grown ones. This could be attributable to the fact that seedlings from the field were subjected to environmental variables such as weeds, water availability and soil microbes. These environmental factors have been previously shown to have a confounding effect on the morphological traits of field-cultivated seedling

(Johnson and Richard, 2005). The root to shoot ratio was higher for all the groups and treatments, especially with the container-grown seedlings from the shadehouse, which were having fibrous roots with massive root hairs that may be due to the soil mixture, which consisted of porous bark and river sand. These increased their surface area for absorption of water and nutrient uptake, which consequently enhanced their relative biomass yields higher than field-grow seedlings.

All the naphthoquinones considered for the study were present in every sample analyzed. However, their mean concentrations were found to be highly variable between groups and among the fertilization treatments applied at both growing sites. Many studies have shown similar responses of secondary metabolites in other species (Kopsell *et al.*, 2004; Almeida-Cortez *et al*., 2004). Environmental differences between the two growing conditions could have influenced the observed chemical variation of naphthoquinones in *Euclea natalensis* seedlings. The concentration of naphthoquinones has been widely reported to vary greatly among populations, within individuals and between plant organs (Repcak *et al*. 2000, Kämäräinen *et al*., 2003). This could be the case with the synthesis of 7-methyljuglone, which regardless of the treatment applied, accumulated at significantly high concentrations in seedlings subjected to the control treatment. Levels of some defensive compounds are directly linked to their ecological significance that can vary depending on their respective bioactivities (Laitinen *et al*., 2005). These results suggest that NPK fertilization can be used to enhance levels of shinanolone, neodiospyrin and isodiospyrin under field conditions.

Previous antimycobacterial activity of *E. natalensis* has only been conducted on strains of *M. tuberculosis* with extracts acquired from mature plants (Lall *et al.*, 2005). Generally, *M. tuberculosis* showed similar sensitivity as *M. smegmatis* (Newton *et al.,* 2002). Conversely, it was found from this study that root extracts from the seedlings were more active, even at lower concentrations on *M. tuberculosis* than *M. smegmatis*. The

lowest MIC value observed previously with crude extracts obtained from mature plants of *E. natalensis* was 0.8 µg/ml (Lall *et al.*, 2005), which is relative to the lowest MIC from the seedlings. The finding shows that a comparative antimycobacterial activity can be achieved with seedlings and their cultivation should therefore be encouraged to conserve wild stocks. Another advantage with seedlings is that they yield other naphthoquinones (isodiospyrin and neodiospyrin) in higher concentrations than in mature plants (unpublished data).

The effect of domestication on the yield of secondary metabolites is well documented (Al-Fayyad *et al*., 2002). Maintaining medicinal plants under controlled environments may significantly reduce their chemical effectiveness by enhancing agronomic qualities. This has been the case in our experiment as seedlings harvested under shadehouse conditions were relatively less active as compared to field-cultivated seedlings under the same treatment.

5.6. CONCLUSION

Our study indicated that subjecting seedlings to controlled environments coupled with higher levels of fertilizers could have a negative impact on the antimycobacterial activity of *E. natalensis*. Field-cultivated seedlings with no application of fertilizers could be useful for optimisation of naphthoquinones necessary for antitubercular studies.

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CHAPTER 6

General discussion and conclusion

6.1. General discussion

The results from the studies showed that the concentration of the naphthoquinones (shinanolone, 7-methyljuglone, diospyrin, isodiospyrin and neodiospyrin) investigated in *Euclea natalensis* vary in response to seedling growth, seasonal changes and application of NPK fertilizers. Qualitative analysis of the chemical profiles from all the samples tested revealed a considerable variation in the type of naphthoquinone between seedlings and mature plants. Many studies conducted with woody plant species have highlighted the transfer of defenses from mainly chemical compounds in seedlings to structural defenses in mature plants (Busk and Møller, 2002; Košuth *et al*., 2003). The qualitative variation in naphthoquinones was also apparent between individuals of *E. natalensis* belonging to the same population.

These studies also demonstrated that naphthoquinones accumulate mostly in the subterranean structures as compared to the aerial parts. Accumulation of high levels of naphthoquinones in belowground biomass, validates the observation that quinones are signal molecules between organisms in the rhizosphere (Wrobel *et. al*., 2002). It also confirms the ethnomedical use of roots harvested by indigenous people of southern Africa. High concentrations of naphthoquinones per plant material were quantified in seedlings as compared to mature plants. Liu *et al*. (1998) hypothesized that certain chemical defenses are programmed for early ontogenic stages, whereas they are induced by biotic and abiotic factors during later developmental stages, which might be the case with naphthoquinone accumulation in *E. natalensis*.

The levels of the specified naphthoquinones examined varied between growth stages, with seasons and among treatments in seedlings of *E. natalensis*. In general, diospyrin was

the only naphthoquinone detected at high mean concentrations from all samples analysed, in seedlings and mature plants. The level of qualitative consistency encountered with diospyrin could be correlated to its ecological significance in *E. natalensis* plant species. Empirical studies indicated that resources are preferentially allocated to chemical defenses that maximize fitness of the species involved.

Accumulation of 7-methyljuglone was highly variable, whereby it was quantified at high mean concentrations in some samples and was not detected in others. Its pattern of metabolite production was clearly apparent in mature plants, where individuals of a population varied significantly during the same season. Kämäräinen *et al*. (2003) found similar differences in the concentration of 7-methyljuglone between various samples of the same populations. The production of 7-methyljuglone was not influenced by fertilizer applications and was quantified at high mean concentration from seedlings under the control treatment. Biosynthesis of 7-methyljuglone may be more dependent on environmental cues than seasonal changes, age factor or fertilization.

Isodiospyrin and neodiospyrin were detected in relatively high mean concentrations from samples acquired from seedlings. Neodiospyrin may be further optimized by application of NPK fertilizers under field conditions. The effect of fertilisation on the levels of secondary metabolites has been widely reported to vary among different plant species as well as the type of secondary compound (see for example, Al-Fayyad *et. al*., 2002; Manukyan, 2005; Guo *et. al*., 2006). These two dimeric naphthoquinones were quantified in trace amounts or not detected from samples obtained from mature plants and it will not be beneficial to target them in plant material acquired from natural populations of *E. natalensis*.

Field-cultivated seedlings of *E. natalensis* exhibited higher antimycobacterial activity against both tested strains as compared to shadehouse seedlings. Their root extracts were even more active on *M. tuberculosis*, showing a level of selectivity against the

antimycobacterial strain. These results suggest that naphthoquinones could be optimized through cultivation of seedlings under field conditions. The study also revealed that fertilizers could lower the antibacterial activity of such seedlings.

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CHAPTER 7

Conference presentations and manuscripts

7.1. Presentations

1. Indigenous Plant Use Forum, Rustenburg, South Africa. (2003) **Paper presentation,** Bapela M J, Lall N, Isaza-Martinez JH, Regnier T and Meyer JJM. Seasonal variation of naphthoquinones in *Euclea natalensis*.

2**.** South African Association of Botanists, University of KwaZulu-Natal, South Africa. (2004) **Oral presentation,** Bapela M J, Lall N, Isaza-Martinez JH, Regnier T and Meyer JJM. Variations in the concentration of naphthoquinones in seedlings of *Euclea natalensis*.

3. South African Association of Botanists, Nelson Mandela Metropolitan University, South Africa**.** (2005) **Oral presentation,** Bapela M J, Lall N and Meyer JJM. Seasonal variation of naphthoquinones in *Euclea natalensis* subspecies *natalensis*.

7.2. Manuscripts resulting from this thesis

1. M.J. Bapela, N. Lall, J.H. Isaza-Martinez, T. Regnier, J.J.M. Meyer (2007). Variation in the content of naphthoquinones in seeds and seedlings of *Euclea natalensis.* South African Journal of Botany (Reprint attached)

2. Seasonal variation of naphthoquinones in *Euclea natalensis* subspecies *natalensis*. M.J. Bapela, N. Lall, J.J.M. Meyer. (Accepted for publication)

3. Fertilization-induced changes in growth parameters and antimycobacterial activity of *Euclea natalensis* (Ebenaceae). (Reprint attached) J. Bapela, V. Kuete, M. Meyer, E. Du Toit, N. Lall.

CHAPTER 8

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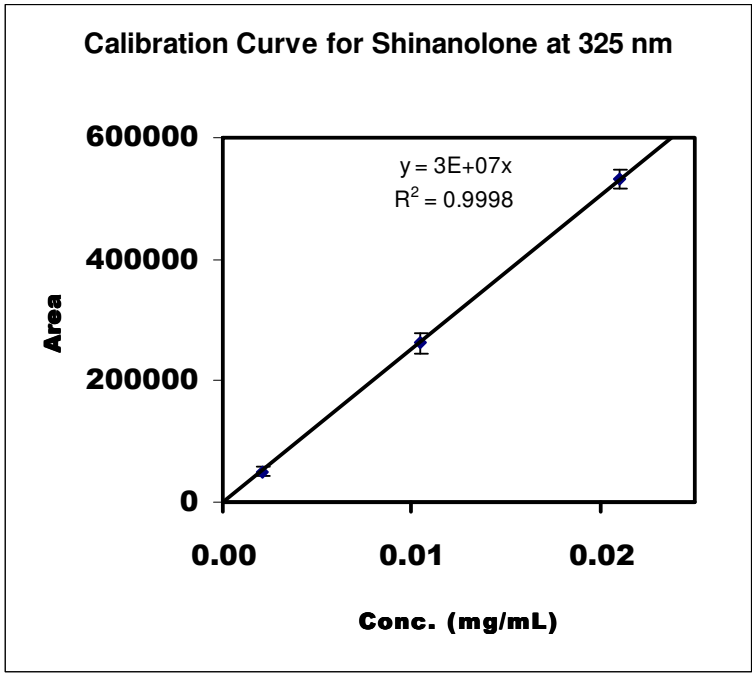
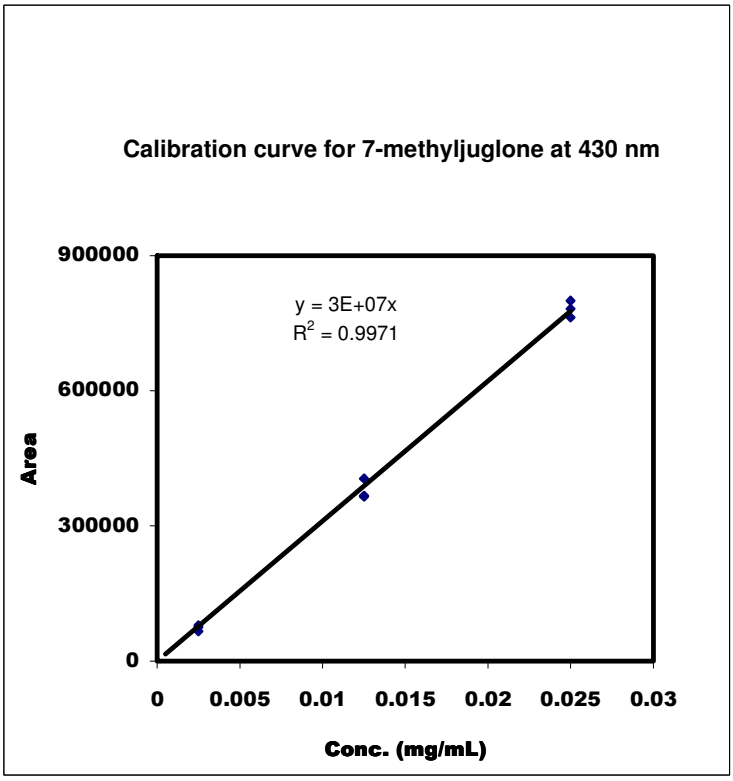
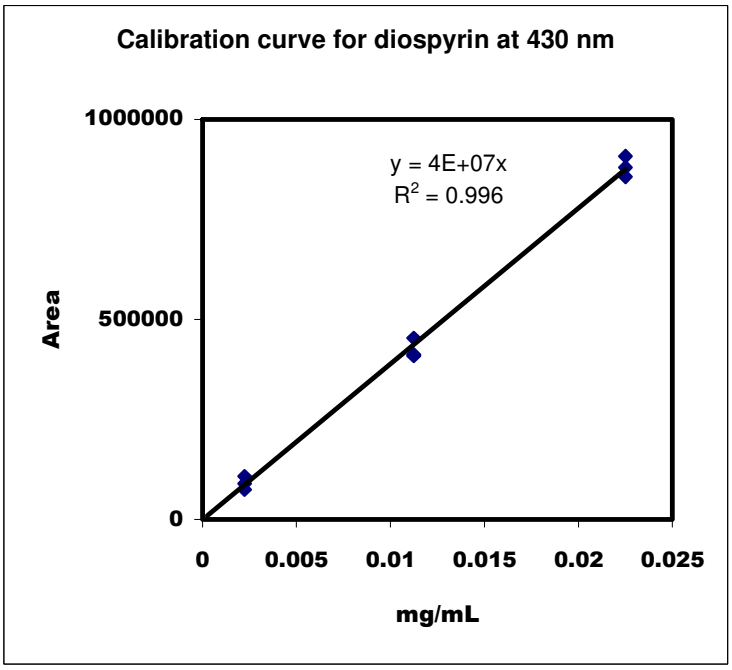
APPENDIX

Standard curves of the quantified naphthoquinones



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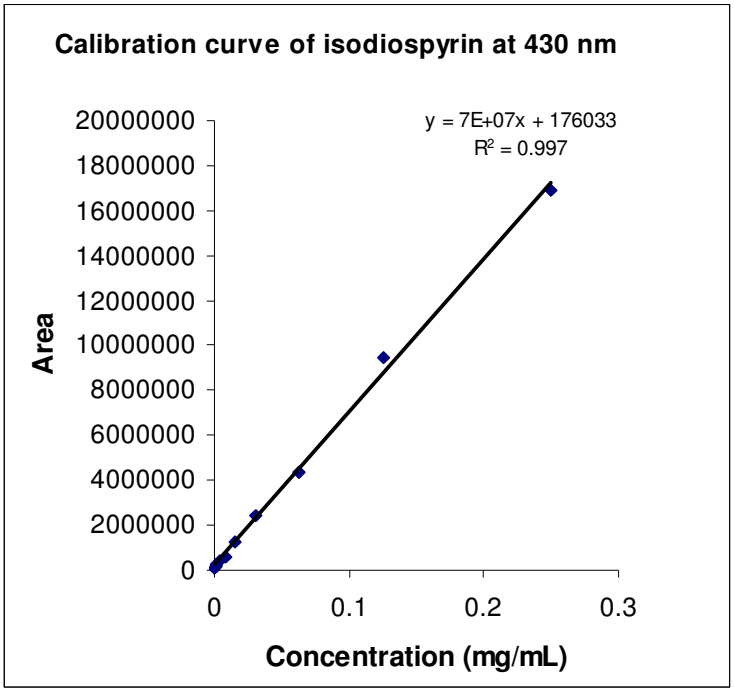
0.005
0.01
0.015
0.02
0.025
mg/mL


Calibration curve of isodiospyrin at 430 nm
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R2 = 0.997
Area
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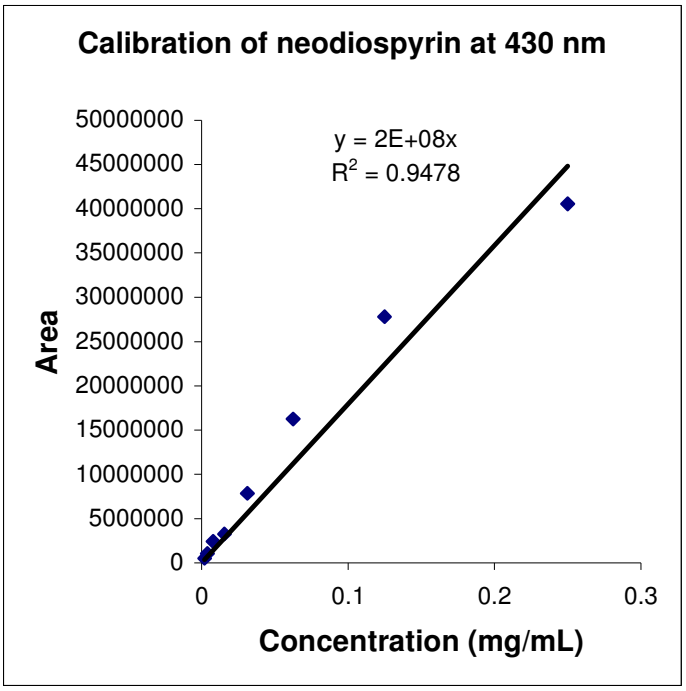
10000000
8000000
6000000
4000000
2000000
0
0
0.1
0.2
0.3
Concentration (mg/mL)


Calibration of neodiospyrin at 430 nm
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R2 = 0.9478
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40000000
35000000
30000000
25000000
20000000
15000000
10000000
5000000
0
0
0.1
0.2
0.3
Concentration (mg/mL)

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Fertilization-induced changes in growth parameters and antimycobacterial activity of *Euclea natalensis* (Ebenaceae)

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Abstract

The effect of Nitrogen: Phosphorus: Potassium (NPK) (2:1:2 (44)) fertilizers on growth, accumulation of bioactive naphthoquinones (shinanolone, 7-methyljuglone, diospyrin, isodiospyrin and neodiospyrin) and antimycobacterial activity was investigated in seedlings of *Euclea natalensis* (Ebenaceae) grown in shadehouse and in field conditions. Seedlings were subjected to three differential fertilization regimes (40 g/l, 20 g/l and 10 g/l). Treatment 2 enhanced the vegetative performance of seedlings grown under shadehouse conditions. A significantly positive correlation was established between the concentration of shinanolone ($P<0.01$), isodiospyrin ($P<0.05$) and neodiospyrin ($P<0.05$) with fertilization from field-grown seedlings. Application of NPK fertilizers significantly ($P<0.05$) increased the accumulation of neodiospyrin in seedlings subjected to shadehouse conditions. The most potent naphthoquinone, 7-methyljuglone was found to be abundant in all the extracts and was quantified at a high mean concentration of 10.2 g/kg from shade-grown seedlings. Extracts acquired from field-grown seedlings were more active against *Mycobacterium smegmatis* with a lowest MIC value of 0.78 mg/ml. Extracts from the control group and Treatment 1 (10 g/l), which had less applied fertilizers, were more active against strains of *M. tuberculosis* with MIC value of 10 μg/ml. This shows the selectivity of *E. natalensis* against *M. tuberculosis*. Our study indicated that subjecting seedlings to homogenous environments coupled with higher levels of fertilizers could have a negative impact on the antimycobacterial activity of *E. natalensis*.

Keywords: Antimycobacterial activity; *Euclea natalensis*; Field; Naphthoquinones; NPK fertilizers; Shadehouse

1. Introduction

The recent resurgence of interests in medicinal plants and plant-derived secondary metabolites is primarily aimed at discovering novel secondary metabolites, which can counteract resistance from pathogens and circumvent side effects that are typically associated with most indispensable therapeutic drugs. Plant material harvested from wild stocks cannot meet the escalating demand of natural products in the growing global market of medicinal and aromatic plants (Nigro et al., 2004). It has become inevitable to guarantee a sustained supply of renewable resources of raw plant materials to both local and global markets by optimization through effective propagation and breeding methods (Jäger and Van Staden, 2000). Introduction of medicinal plants into cultivation will not only ensure a continued supply of phytochemicals, but will also ascertain conservation of genetic resources from wild populations. This will alleviate their over-exploitation and relieve the pressure exerted on wild plant species.

Generally, plants synthesize and accumulate secondary metabolites in response to the pressure exerted by biotic and abiotic ecological factors found within their immediate environment. If such interactions are broken, then these plants species are most likely to only yield trace amounts of the compounds of interest. Empirical studies have revealed that domestication of wild species, which involves optimization of growth conditions, may affect their allocation patterns resulting in trade-offs between

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agronomical traits and chemical qualities (Máthé, 1988). Cultivation trials necessitate parallel studies, which are mainly aimed at elucidating factors such as exogenous application of elicitors, fertilizers and soil properties that have been shown to affect plant productivity and quality. The medicinal attributes of each harvest need to be systematically evaluated, using fingerprinting techniques and antimicrobial assays to assure standardization and safety of extracted phytochemicals (McGaw et al., 2002).

Euclea natalensis A.DC., a member of the Ebenaceae family, is endowed with a broad variety of medicinal and horticultural qualities. Many *Euclea* species are frequently gathered by indigenous people of southern Africa to treat respiratory complications such as chest pains, bronchitis, pleurisy and asthma (Bryant, 1966). The ground powder of its roots is topically applied to the skin in cases of leprosy and used by some ethnic groups to relieve toothache and headache (Palgrave, 1991). During pharmacological evaluation of crude extracts and naphthoquinones (shinanolone, 7-methyljuglone, diospyrin, isodiospyrin and neodiospyrin) isolated from *E. natalensis*, it was found that these compounds have antibacterial activity against drug-resistant and drug-sensitive strains of *Mycobacterium tuberculosis* (Lall and Meyer, 2001; Lall et al., 2005). Some of these bioactive naphthoquinones accumulate in minute amounts, and are not available in synthetic form. As a result more plant material is required for further studies such as pre-clinical and clinical trials. This prompted us to conduct a study in an attempt to assess the effect of fertilizers on the synthesis and accumulation of these antitubercular compounds in seedlings of *E. natalensis*, cultivated in field setting and under shadehouse conditions. The bioactivity of crude extracts from the seedlings of *E. natalensis* was evaluated against strains of *M. smegmatis* and *M. tuberculosis*.

2. Materials and methods

2.1. Study area

Cultivation trials were conducted at the Experimental Farm of the University of Pretoria, South Africa, during November 2003–August 2005. The soil of the experimental site is well drained and sandy loam in texture (sand 62.9%, silt 22.9% and clay 7.1%), with a pH of 7.6 and available P, Ca, K, Mg and Na at 102.9, 2854.7, 258.7, 397.3 and 49 mg/kg respectively.

2.2. Plant material

Seeds of *E. natalensis* A.DC. were collected from their natural populations in Tembe Elephant Park, KwaZulu-Natal, South Africa in 2002. A voucher specimen (PRU91601) was authenticated and deposited at the H.G.W.J. Schweikerdt Herbarium, University of Pretoria. The seeds were germinated in growth chambers set at 30 °C and exposed to a cycle of 16 h dark and 8 h light. After radicle emergence, seedlings were transplanted into pots with a soil mixture of river sand: topsoil: compost (bark) (1:1:2) and then kept in a shadehouse while waiting for the 3–4 true leaf stage. After 10 months of establishment the seedlings were divided into two groups, one of which remained under the shadehouse, while the other group was transplanted to the field. The experimental layout was a randomised block design and the seedlings were planted at a space of 30 cm apart in the field.

Each group was further subdivided into four subgroups, which were then subjected to four respective treatments. Water-soluble foliar feed NPK (2:1:2) was applied at three different concentrations. Treatments tested were Treatment 1 (40 g/l), Treatment 2 (20 g/l) (suppliers' recommended concentration) and Treatment 3 (10 g/l). The control group received only supplemental water. Treatments were applied at 200 ml per plant once in every 2 weeks for one year. Treatments were the same for both shadehouse and field seedlings. Irrigation was carried out three times per week in the morning (8 am). The first harvest was conducted after 6 months of application of fertilizers. The second plant collection was done after 12 months of treatment. Leaves were excised from the aerial parts since leaves are not used by indigenous people for healing. Heights, fresh and dry weight of the seedlings were measured for their vegetative performance. Roots of seedlings were dried at room temperature and then ground to yield fine powder for further analysis.

2.3. Extraction

Three replicates of 100 mg of each powder were weighed and then extracted three times, for 48 h using 500 ml of chloroform, and filtered under vacuum. The filtrates were evaporated under nitrogen (Reacti-Vap, model 18780) to yield dry crude extracts.

2.4. Phytochemical analysis

Part of the crude extracts (0.1 mg) was dissolved in acetonitrile (2 ml) and sonicated (Elma Transsonic Waterbath) for 5 min. Dissolved extracts were centrifuged (microcentrifuge Millipore) and subjected to quantitative analysis with a High Performance Liquid Chromatography (HPLC). Chromatographic analysis was carried out using Varian 9012 chromatograph equipped with a diode array detector UV6000LP, and kept at a constant temperature of 25 °C (Joubert et al., 2006). The column used was Phenomenex Luna C18 (2) 3 µ, 150 × 4.6 mm, S/N 111577-5. A mobile phase of acetonitrile (MeCN): water (H_2O): acetic acid (AcOH) (62.5: 32.5: 5) was used in an isocratic mode at a flow rate of 0.8 ml/min and the run time for each injection was 22 min. The sample injection volume was 10 µl and three replicates were conducted.

Naphthoquinones (Fig. 1) were identified by comparison with the retention time and UV spectra of purified standards (Joubert et al., 2006). For quantification of the naphthoquinones present in the samples, each pure compound was dissolved and diluted to yield a series of concentrations in the range of 2.25 µg/ml to 22.5 µg/ml, which were analysed by HPLC as described above (Joubert et al., 2006). The wavelength used for detection was 430 nm for 7-methyljuglone, diospyrin, isodiospyrin and neodiospyrin, and 325 nm for shinanolone. Calibration curves were then generated for each compound

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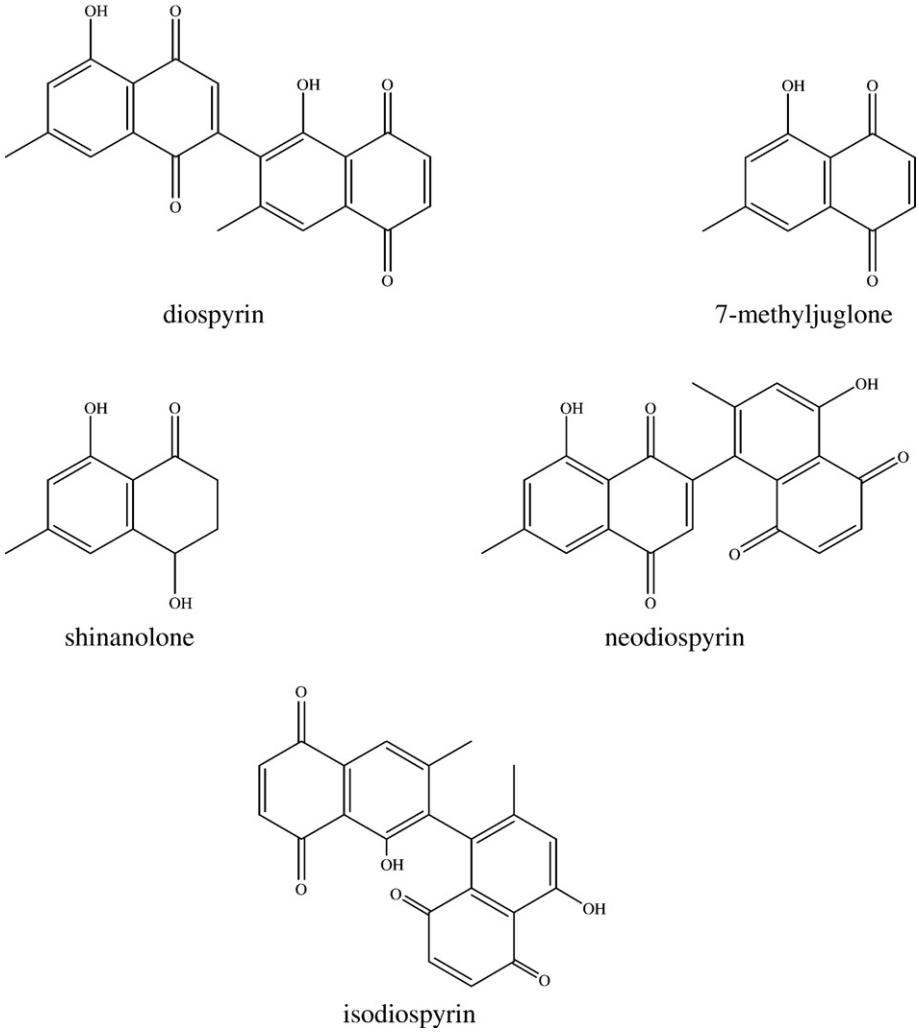

Fig. 1. Naphthoquinones quantified from seedlings of *Euclea natalensis*.

using linear least-square regression equations derived from the resulting peak areas.

2.5. Tested microorganisms

The microorganisms, *Mycobacterium smegmatis* (MC^2 155) and *M. tuberculosis*, H37Rv (ATCC 27264) were obtained from American Type, MD, USA Culture Collection. *M. smegmatis* was cultured and maintained on Middlebrook 7H11 agar base (Sigma-Aldrich Chemical Co., South Africa) for 24 h at 37 °C. *M. tuberculosis* was plated onto Löwenstein-Jensen medium and allowed to grow for 3–4 weeks at 37 °C.

2.6. Microdilution screening assay using M. smegmatis

M. smegmatis colonies were transferred from the surface of the agar base into Middlebrook 7H9 broth base (Sigma-Aldrich Chemical Co., South Africa), which was supplemented with Oleic-Albumin Dextrose-Catalase (OADC). The suspension was homogenized with a vortex mixer (Heidolph, Germany) and allowed to settle for 30 min. The supernatant was collected and adjusted with Middlebrook 7H9 broth base to an optical density of 0.2 (log-phase) at 550 nm (Beckman DU-720 UV spectrophotometer). The bacterial concentration of the suspension was 1.26×10^8 colony-forming units per millilitre (CFU/ml) at the beginning of the test (Mativandlela et al., 2006).

The crude extracts were each prepared by first dissolving in 100% Dimethyl Sulfoxide (DMSO) and then adjusted to 10% DMSO with Middlebrook 7H9 broth base to yield stock solutions with a concentration of 100 mg/ml. Growth control experiments on *M. smegmatis* indicated that a concentration of 2.5% DMSO/well or less did not inhibit the bacterial growth. The microdilution assay was conducted in 96-well microtitre plates using Middlebrook 7H9 broth base as a blank at 100 µl/well (Newton et al., 2002). For the test, 100 µl of each 10% DMSO, stock solution from the crude extracts, ciprofloxacin and broth base were added to the first rows of the plates. Ciprofloxacin served as the positive control drug with a concentration range of 0.31 to 9.76 mg/ml (Salie et al., 1996). DMSO and the culture medium were used as the solvent control and growth control respectively. The experiment was done in triplicate. Serial two-fold dilutions of each sample to be tested were prepared with Middlebrook 7H9 broth base to yield volumes of 100 µl/well with final concentrations of extracts

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ranging from 0.39 to 25 mg/ml. The bacterial suspension was added to each well at 100 µl to give a final volume of 200 µl/well and the plates were incubated at 37 °C for 24 h.

The minimum inhibitory concentration (MIC) of each extract was determined by adding 40 µl of 0.2 mg/ml of *p*-iodonitrotetrazolium chloride (INT, Sigma-Aldrich Chemical Co., South Africa) and incubating at 37 °C for 1 h (Eloff, 1998). Viable bacteria reduced the yellow dye to a pink colour. MIC was defined as the lowest sample concentration that prevented this chemical reduction and displayed absolute inhibition of the bacterial growth. The minimum bactericidal concentration (MBC) was determined by adding 50 µl of aliquots from wells that did not show any growth after incubation during the MIC assay, to 150 µl of broth. These were incubated for 48 h and the viability of *M. smegmatis* was tested as in the MIC assay. MBC was regarded as the lowest concentration of extract, which did not prevent bacterial growth after 48 h of incubation.

2.7. Antitubercular rapid radiometric assay using *M. tuberculosis*

The antitubercular activity of the crude extracts obtained from the first and the second harvest of *E. natalensis* seedlings was conducted using the BACTEC (Becton Dickinson Diagnostic Instrument, Sparks, md) radiometric respiratory technique as previously prescribed (Lall and Meyer, 2001; Mativandlela et al., 2006). Crude extracts were dissolved in DMSO to yield stock solutions of 5 mg/ml. Control experiments showed that a final concentration of 1% DMSO in the medium had no adverse effect on the growth of *M. tuberculosis*. All the extracts were tested at concentrations ranging from 5 to 25 µg/ml based on their respective MIC values on *M. smegmatis*. Three concentrations were assayed in duplicate for each test sample. The primary drug isoniazid (INH) (Sigma-Aldrich, South Africa) was used as a drug-control at a final concentration of 0.2 µg/ml.

A homogenous culture (0.1 ml of *M. tuberculosis*, yielding 1×10^4 to 1×10^5 CFU/ml) was inoculated into the vials containing extracts as well as those of the controls. Two growth control groups were assayed in triplicate. The initial inoculum and 1% DMSO were added to the first growth control replicates. In the second growth control group, each vial was inoculated with 100 µl of a 1:100 dilution of the inoculum to produce an initial concentration representing 1% of the bacterial population (1×10^2 to 1×10^3 CFU/ml). The MIC was defined as the lowest concentration of the extract that inhibited >99% of the bacterial population. *Mycobacterium* growing in 7H12 medium containing ^{14}C-labelled substrate (palmitic acid) uses the substrate and produces $^{14}CO_2$. The amount of $^{14}CO_2$ detected is expressed in terms of the growth index (GI) (Middlebrook et al., 1977). Inoculated bottles were incubated at 37 °C and each bottle was assayed daily to measure GI, until cumulative results were interpretable (Lall and Meyer, 2001).

2.8. Statistical analysis

Statistical analyses were performed by means of the SAS (SAS Institute, Inc, Cary, NC, Version 7.01, USA) Programme. The mean values obtained from different groups were compared by one-way ANOVA, assuming the differences between the means were significant at $P<0.05$, $P<0.01$ and $P<0.001$.

3. Results

3.1. Vegetative performance

Vegetative performance of *E. natalensis* seedlings in relation to fertilizer treatments during the growing season of November 2003 and August 2005 is shown on Table 1. Growth parameters differed between the two groups and between treatments, with seedlings from the shadehouse showing more plant vigour than the field-grown plants. No significant correlation could be established between treatment and the measured growth factors from field-grown seedlings. Mean values of seedlings from the shadehouse during the second harvest were two-fold or higher than those of field seedlings. A significant variation ($P<0.001$)

Table 1
Mean values[a] of growth parameters of *Euclea natalensis* seedlings grown under field and shadehouse conditions

Harvest	Treatment	Field seedlings					Shadehouse seedlings				
		Height (cm)	FWS (g)	FWR (g)	DWS (g)	DWR (g)	Height (cm)	FWS (g)	FWR (g)	DWS (g)	DWR (g)
First	Control	30.0±4.4	0.8±0.4	4.3±1.8	0.7±0.3	3.1±1.6	30.1±4.7	2.9±1.6	15.9±3.5	1.6±0.9	9.8±2.8
	Treatment 1	32.3±4.7	1.5±0.1	8.7±1.9	1.3±0.1	6.1±1.5	30.1±6.4	1.6±0.6	15.8±0.7	1.8±0.2	9.0±2.0
	Treatment 2	25.3±3.5	2.2±0.5	7.3±5.6	1.4±1.2	4.9±3.4	36.0±1.0	5.3±0.6	21.4±8.3	2.6±0.3	9.7±3.8
	Treatment 3	28.0±3.6	1.4±1.0	6.3±0.7	1.3±0.9	4.3±0.4	34.7±7.5	5.8±1.9	19.5±6.6	2.9±0.9	6.8±5.6
Second	Control	39.0±4.0	3.7±1.5	9.6±4.4	2.2±0.9	6.2±2.9	93.3±6.7	4.1±2.5	20.9±3.4	2.1±0.3	13.5±3.3
	Treatment 1	48.7±3.1	3.3±0.3	10.4±1.3	1.9±0.2	6.2±2.2	66.0±6.2	4.8±1.1	19.5±3.3	2.1±0.4	15.6±2.0
	Treatment 2	49.0±6.6	2.4±2.0	8.7±2.2	1.8±0.3	5.9±1.1	120.0±8.0	6.3±1.3	32.1±15.2	3.7±0.8	20.1±1.8
	Treatment 3	46.0±9.0	2.6±0.9	12.1±1.6	1.6±0.5	7.7±1.5	94.0±7.0	6.8±1.4	30.4±11.2	3.6±1.1	23.9±2.9
Significance[b] due to:											
Harvest		***	**	*	*	*	***	*	*	*	***
Treatment		NS	NS	NS	NS	NS	***	**	*	**	**
Harvest X Treatment		NS	NS	NS	NS	NS	***	NS	*	NS	NS

[a]Each mean value is an average of three replications±standard deviation. [b]By analysis of variance: ***, P<0.001; **, $P<0.01$; *, $P<0.05$; NS, not significant. FWS=Fresh weight of shoots, FWR=Fresh weight of roots, DWS=Dry weight of shoots, DWR=Dry weight of roots.

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Table 2
Total yields of crude extracts from seedlings of *Euclea natalensis*

Harvest	Treatment	Field	Shadehouse
		Mass in (g/plant material) and in (%) (in parenthesis)	Mass in (g/plant material) and in (%) (in parenthesis)
First	Control	0.35 (2.1)	0.24 (0.5)
	Treatment 1	0.47 (2.9)	0.53 (2.1)
	Treatment 2	0.50 (3.9)	0.48 (1.9)
	Treatment 3	0.41 (3.8)	0.54 (2.0)
Second	Control	1.17 (6.5)	0.55 (2.3)
	Treatment 1	0.63 (3.9)	0.21 (2.1)
	Treatment 2	0.62 (4.0)	1.01 (2.3)
	Treatment 3	0.67 (4.1)	1.07 (3.4)

was observed between treatment and the growth parameters of shadehouse seedlings.

Treatment 2, which is the recommended dosage, enhanced vegetative performance with the mean values of fresh weight of shoots and roots twice as much as their respective control mean values. A significant variation ($P<0.001$) was also found between harvest and treatment from shadehouse seedlings, as more biomass was collected from the second harvest. Seedlings harvested from the shadehouse under Treatment 2 had higher mean values for all the vegetative parameters measured and differed considerably from the field seedlings under the same treatment.

3.2. Extraction yields

Table 2 shows the comparative analysis of the crude extracts obtained from the first and the second harvest in seedlings of *E. natalensis* grown under field and shadehouse conditions. Seedlings harvested from the control group under field conditions yielded the highest percentage of crude extract per plant material (6.5%) in all the groups. The percentages of crude extract per plant material obtained from the field-cultivated seedlings were always higher than their corresponding treatments from the shadehouse seedlings.

3.3. Phytochemical analysis

Table 3 shows the comparative analysis of the effect of NPK fertilizers on the concentration of active constituents of *E. natalensis*. A positive correlation was established between the concentration of shinanolone ($P<0.01$), isodiospyrin ($P<0.01$) and neodiospyrin ($P<0.05$) with fertilizer treatments in field-grown seedlings. Shinanolone and neodiospyrin were quantified at high mean values of 4.5 g/kg and 3.2 g/kg respectively, from seedlings subjected to Treatment 1 under field conditions during the second harvest. Treatment 2 enhanced the accumulation of isodiospyrin for both groups. There was no significant difference detected from the respective mean values of 7-methyljuglone and diospyrin with treatments in field-cultivated seedlings, although their levels increased with time. A significant increase ($P<0.05$) in the concentration of neodiospyrin was observed in shadehouse seedlings.

A negative correlation ($P<0.001$) was found between the accumulation of 7-methyljuglone and fertilizer treatment under shadehouse conditions. It is worth noting that 7-methyljuglone, the most abundant and potent (Lall et al., 2005) naphthoquinone, was quantified at a highest mean value (10.2 g/kg) in samples acquired from control groups under shadehouse conditions during the second harvest. The second harvest yielded a relatively high concentration of naphthoquinones except for neodiospyrin, in which the first harvest was mostly two-fold the amount acquired from the second harvest.

3.4. Antimycobacterial activity of E. natalensis seedlings

The antimycobacterial activity of root extracts from seedlings of *E. natalensis* obtained during the first and the second harvests is illustrated in Table 4. Root extracts of *E. natalensis* seedlings grown under field conditions were generally more

Table 3
The concentration[a] of naphthoquinones in field-grown and shadehouse seedlings of *Euclea natalensis*

Harvest	Treatment	Field seedlings					Shadehouse seedlings				
		Shinanolone (g/kg)	7-Methyljuglone (g/kg)	Diospyrin (g/kg)	Isodiospyrin (g/kg)	Neodiospyrin (g/kg)	Shinanolone (g/kg)	7-Methyljuglone (g/kg)	Diospyrin (g/kg)	Isodiospyrin (g/kg)	Neodiospyrin (g/kg)
First	Control	2.1±2.2	6.4±4.2	3.7±1.7	0.24±0.2	2.4±2.8	2.6±2.7	4.6±4.9	4.4±3.1	0.8±0.8	1.1±1.2
	Treatment 1	3.2±3.3	6.6±7.1	2.5±1.8	0.23±0.6	3.2±3.6	1.5±2.1	3.4±3.9	6.8±5.7	0.8±0.8	0.7±0.9
	Treatment 2	2.7±2.9	3.6±3.8	2.8±1.8	0.61±0.5	2.5±2.8	3.2±3.4	2.0±3.1	5.7±4.2	0.7±1.0	1.4±1.9
	Treatment 3	1.8±1.9	3.6±4.2	2.3±1.3	0.34±0.2	1.6±2.0	3.4±3.5	2.7±2.8	4.1±3.2	0.7±0.4	1.4±1.8
Second	Control	4.1±2.3	6.9±4.9	3.0±2.3	0.3±0.2	1.2±1.5	3.3±3.7	10.2±11.1	5.3±3.9	0.8±0.7	0.9±1.2
	Treatment 1	4.5±4.6	6.5±7.1	3.6±2.2	0.4±0.3	1.7±2.1	3.7±4.1	4.8±5.7	5.9±3.9	1.2±1.1	0.4±4.2
	Treatment 2	3.8±4.0	5.6±6.0	3.7±4.6	0.8±1.3	1.8±1.9	4.9±4.2	8.2±9.1	5.4±4.7	0.6±0.6	2.1±2.8
	Treatment 3	2.5±2.3	4.1±4.4	2.5±0.9	0.4±0.2	1.0±1.1	4.1±4.2	6.3±6.9	5.9±5.6	1.0±1.1	0.5±0.6
Significance[b] due to:											
Harvest		NS	NS	NS	NS	**	**	***	NS	NS	NS
Treatment		**	*	NS	**	*	NS	**	NS	NS	*
Harvest X Treatment		NS	NS	NS	NS	NS	NS	*	NS	NS	*

[a]Each concentration represents a mean value of three replications±standard deviation. [b]By analysis of variance: ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; NS, not significant.

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Table 4
Antimycobacterial activity of *Euclea natalensis* seedlings

Seedlings	Treatment	*M. smegmatis*				*M. tuberculosis*			
		First harvest		Second harvest		First harvest		Second harvest	
		[a]MIC (mg/ml)	[b]MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (μg/ml)	ΔGI[c]	MIC (μg/ml)	ΔGI[b]
Field	Control	6.25	25	0.78	12.5	20	0.0±0.0	10	9.0±0.0
	Treatment 1	6.25	NA*	0.78	12.5	20	23±4.24	10	0.5±0.5
	Treatment 2	6.25	25	1.56	25	25	9.5±0.71	15	1.5±3.5
	Treatment 3	6.25	25	3.13	25	25	7±4.24	15	0.5±0.7
Shadehouse	Control	12.5	NA*	3.13	25	20	1±0	10	−2.0±0.0
	Treatment 1	6.25	25	1.56	12.5	25	10.5±0.71	15	0.0±0.0
	Treatment 2	25	NA*	6.25	25	25	6±1.41	15	1.5±0.7
	Treatment 3	6.25	25	3.13	25	25	14.5±4.95	15	0.5±0.7
RA		0.61	1.22	0.61	1.22	0.12	−6.50±0.72	0.12	−6.50±0.72

[a]Minimum inhibitory concentration, [b]Minimum bactericidal concentration, [c]ΔGI value (mean±standard deviation) of the control vial was 29,5±0.71 for the sensitive strain. NA*; no activity, RA: reference antibiotics, ciprofloxacin and isoniazid for *M. smegmatis* and *M. tuberculosis* respectively.

active against *M. smegmatis* than extracts acquired from roots of seedlings maintained under shadehouse conditions. The lowest MIC of 0.781 mg/ml was obtained from the control and Treatment 1 seedlings, which were collected during the second harvest under field conditions. These extracts had high levels of 7-methyljuglone, which is known to be highly active (Lall et al., 2005). A relatively higher MIC of 3.13 mg/ml was found in field-grown seedlings subjected to Treatment 3 from the second harvest. The bioactivity of root extracts acquired from shadehouse seedlings during the second harvest was comparatively lower than those of field plants and the MIC ranged from 1.56 to 6.25 mg/ml. MBC values for both groups during the second harvest were higher and recorded between 12.5 and 25 mg/ml. The second harvest yielded more active extracts than the first harvest, probably due to the increase of bioactive compounds (Table 4). The MIC values of the crude extracts during the first harvest were higher and ranged from 6.25 to 25 mg/ml. Their respective MBC values were even higher and in some instances there was no activity at all.

Field-grown seedlings showed a higher antitubercular activity than the seedlings grown in the shadehouse, as can be seen in the *M. smegmatis* experiment (Table 4). There appears to be a high level of selectivity of *E. natalensis* activity towards *M. tuberculosis* as the extracts were active at lower concentrations. Extracts from the control group and Treatment 1, which had less fertilizer were more active against strains of *M. tuberculosis* with a MIC value of 10 μg/ml. The shadehouse conditions did not enhance the antimycobacterial activity of the root extract.

4. Discussion

Biomass production increased significantly as a result of fertilizer applications. Seedlings from the shadehouse displaying improved vegetative performance compared to field-grown ones. This could be attributed to the fact that field seedlings were subjected to a host of environmental variables such as weeds, water availability and soil microbes. These environmental factors were previously shown to have a confounding effect on the morphological traits of field-cultivated seedling (Johnson and Richard, 2005). The root to shoot ratio was higher for all the groups and treatments, especially with the container-grown seedlings from the shadehouse, which had fibrous roots with massive root hairs that may be due to the soil mixture that consisted of porous bark and river sand. These increased their surface area for absorption of water and nutrients, which consequently enhanced their relative biomass yields higher than field harvested seedling. All the naphthoquinones considered for the study were present in every sample analyzed. However, their mean concentrations were found to be highly variable between groups and among the different fertilizer treatments at both growth sites. Previous studies have shown similar responses of secondary metabolite production in other species (Kopsell et al., 2004; Almeida-Cortez et al., 2004). Environmental differences between the two growing locations could have influenced the observed chemical variation of naphthoquinones in *E. natalensis* seedlings. It is known that the concentration of naphthoquinones can vary greatly among populations, within individuals and between plant organs (Repcak et al., 2000; Kämäräinen et al., 2003). This could also be the case with the synthesis of 7-methyljuglone, which, regardless of the treatment applied, accumulated significantly from the first to the second harvest in control seedlings grown in the shadehouse. The levels of some defense compounds are directly linked to their ecological significance that can vary depending on their respective bioactivities (Laitinen et al., 2005). The results of this study suggest that NPK fertilization can be used to enhance levels of shinanolone, neodiospyrin and isodiospyrin under field conditions.

Previously, antimycobacterial activity of *E. natalensis* on *M. tuberculosis* has only been conducted with extracts acquired from mature plants (Lall et al., 2005). Generally, *M. tuberculosis* showed similar sensitivity as *M. smegmatis* (Newton et al., 2002). However, it was found in this study that root extracts from the seedlings were more effective against *M. tuberculosis* than *M. smegmatis*. The lowest MIC value observed previously with crude extracts obtained from mature plants of *E. natalensis* was 0.8 μg/ml (Lall et al., 2005), which

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is to the lowest MIC from the seedlings. The finding shows that a comparative antimycobacterial activity can be achieved with seedlings and their cultivation should therefore be encouraged to conserve wild stocks. Another advantage with seedlings is that they yield other naphthoquinones (isodiospyrin and neodiospyrin) in higher concentrations than mature plants (unpublished data).

The effect of domestication on the yield of secondary metabolites is well documented (Al-Fayyad et al., 2002). Maintaining medicinal plants under controlled environments may significantly reduce their chemical effectiveness by enhancing agronomic qualities. This has been the case in our experiment as seedlings grown in the shadehouse were less active compared to field-cultivated seedlings with the same treatment. This study indicates that subjecting seedlings to controlled environments coupled with higher levels of fertilizers could have a negative impact on the antimycobacterial activity of *E. natalensis*. Field-cultivated seedlings with no application of fertilizers could be useful for optimisation of the production of naphthoquinones required for antitubercular studies.

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Variation in the content of naphthoquinones in seeds and seedlings of *Euclea natalensis*

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Abstract

A correlation between plant growth and accumulation of naphthoquinones (shinanolone (**1**), 7-methyljuglone (**2**) and diospyrin (**3**)) was investigated in seeds and seedlings of *Euclea natalensis* A.DC. In this study, the seeds represented the first stage whereas the second seedling stage was defined as the stage, when the radicles were about 6 cm in length. The lengths of the seedlings at the third, fourth and fifth seedling stages were 9 cm, 12 cm and 16 cm respectively. Plant materials collected from the five seedling stages were separately extracted using chloroform and the naphthoquinones were then quantified by means of High Performance Liquid Chromatography (HPLC). Shinanolone (**1**), which was the only naphthoquinone detectible from seeds, accumulated at variable rates ($P<0.01$) and no trend could be established between its synthesis and seedling growth. The content of shinanolone (**1**) ranged from 87.5 mg/kg in seeds (first stage) to a high mean value of 1047 mg/kg during the fourth seedling stage. A significant correlation ($P<0.01$) was found between the mean concentrations of 7-methyljuglone (**2**) and seedling growth. 7-Methyljuglone (**2**) was quantified at a high mean level of 5003 mg/kg during the third seedling stage and was not detected from the seed samples. A positive correlation ($P<0.01$) was established between the concentration of diospyrin (**3**) and seedling stages. Diospyrin (**3**) was detected at an elevated mean concentration of 6182 mg/kg during the fifth seedling stage, which was higher than the other quantified naphthoquinones.

Keywords: Ebenaceae; *Euclea natalensis*; Naphthoquinones

1. Introduction

Euclea natalensis A.DC., a member of the Ebenaceae family, is extensively distributed along the eastern coast of southern Africa extending inland into Swaziland and Botswana (Van Wyk and Gericke, 2000). It grows as a shrub to a medium sized tree of about 12 m in height, often with a spreading crown and occurs in a wide variety of habitats (Palgrave, 1991). Southern African natives use the roots of *E. natalensis* for a number of medicinal purposes. These include the preparation of purgatives, topical application to skin lesion in cases of leprosy (Palgrave, 1991) relief of toothache, headache and chest complaints (Van Wyk and Gericke, 2000). The pharmacological activity of *E. natalensis* is attributed to, among other compounds, the presence of naphthoquinones, which are the dominant secondary metabolites of the Ebenaceae family (Bryant, 1966; Van Wyk and Van Wyk, 1997). Naphthoquinones are allelopathic, antiherbivorous and endowed with antimicrobial properties against a broad spectrum of pathogens (Ioset et al., 1998; Sasaki et al., 2002).

Previous investigations on the pharmacological activity of naphthoquinones revealed their effectiveness against protozoan infections such as leishmaniasis and malaria (Hudson et al., 1998; Kayser et al., 2000). Several naphthoquinones, including 7-methyljuglone (**2**), diospyrin (**3**), isodiospyrin, shinanolone (**1**) and mamegakinone have already been isolated from many species of the Ebenaceae family (Van der Vijver and Gerritsma, 1974; Ferreira et al., 1977; Khan, 1985). Diospyrin (**3**) and

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isodiospyrin, which are phytochemical constituents of *Diospyros piscatorial*, showed a wide spectrum of antibacterial activity (Adeniyi et al., 2000). Three naphthoquinones (diospyrin (**3**), 7-methyljuglone (**2**) and shinanolone (**1**)) isolated from *E. natalensis* exhibited significant activity against drug-sensitive and drug-resistant strains of *Mycobacterium tuberculosis* (Lall and Meyer, 2000). Synergistic studies of these naphthoquinones with existing TB drugs and clinical trials are currently underway and therefore a substantial amount of bioactive naphthoquinones is required.

The type and level of naphthoquinones vary between different plant species and variation in bioactivity is often encountered between different parts of the same plant. In *Drosera* species, the amount of naphthoquinones varies interspecifically (Bonnet et al., 1984), in different tissues of the plant (Repcak et al., 2000) and during the growing season (Caniato et al., 1989). Some of these compounds are not available in synthetic form, and harvest timing is not a viable option given the spatial and temporal variation of naphthoquinone production. Sustainability can be achieved through effective breeding strategies and studies should therefore, be conducted to evaluate the effect of domestication on the concentration of secondary metabolites in medicinal plants (Appleton and Van Staden, 1995). This study was conducted to determine the presence of naphthoquinones in seeds and variation in the content of three naphthoquinones in seedlings of *E. natalensis* grown under shadecloth.

2. Materials and methods

2.1. Germination procedure and samples

Matured seeds were collected from natural populations of *E. natalensis* at Tembe National Park, KwaZulu Natal, RSA, in 2001. A voucher specimen (PRU: 91601) was deposited at the H.G.W.J. Schweikerdt Herbarium, University of Pretoria. Pericarps of fruits were manually removed and only naked seeds were used in the experiment. Approximately 500 seeds of *E. natalensis* were sterilized with 10% sodium hypochlorite solution for 5 min and then rinsed five times with autoclaved distilled water. Ten Perspex boxes were then sterilized with 100% ethanol and allowed to dry in a laminar flow. Thereafter, three layers of moist autoclaved cellulose wadding were laid in each box.

Fifty seeds per box were dispersed randomly on the wet cellulose wadding and incubated in growth chambers at 30 °C and exposed to a cycle of 16 h light and 8 h dark. Monitoring was conducted on a daily basis and water replenished when necessary. The seedlings were harvested at four different growth stages using shoot and root length as variables. For the purpose of this experiment, the dormant stage, which consisted of seeds, was referred to as stage 1. Radicle emergence begun after 5 weeks of incubation.

Three hundred seedlings were harvested at the second seedling stage, which was identified as the point after root protrusion when the radicles were darkened in colour and were about 6 cm in length. The remaining seedlings were transplanted into individual pots containing sterile compost and transferred to a greenhouse.

The seedlings of *E. natalensis* are classified as epigeal, indicating that the cotyledons are aboveground and photosynthetic (Mayer and Poljakoff-Mayber, 1982). Consequently, hypocotyls were harvested separately from the roots, at the third stage when they consisted of single stems with cotyledons embedded within the seed coats. The hypocotyls had an average length of 1 cm and the roots were 8 cm in length. Seedlings with two photosynthesizing cotyledons each characterized the fourth growth stage and like in the previous stage, the aerial parts were separated from subterranean parts at the base of the hypocotyls. The shoots were 2 cm and the roots were 10 cm in length. The fifth growth stage consisted of seedlings with 3–5 true leaves with the length of the shoots being 3 cm whereas the roots measured 13 cm. Leaves were excised from the aerial parts at the base of their petioles. Dried seeds, shoots and roots harvested from the four seedling stages, constituted samples for analysis. All samples were air-dried at room temperature, and ground to yield homogenous powders using a Junke & Kunkel, Funkentstort KB5/10 (analysing miller).

2.2. Phytochemical analysis

Approximately 100 mg of ground seeds from the first stage and 100 mg of radicles from the second stage of the seedlings of *E. natalensis* were weighed out for analysis. Hundred milligrams of roots and shoots from the third, fourth and fifth seedling stages of *E. natalensis* were also weighed out. All these samples were extracted (three times at two-hour interval) using 2 ml of chloroform and then filtered under vacuum. The chloroform extracts were then evaporated using a nitrogen unit (Reacti-Vap, model 18780) to yield dry crude extracts. Qualitative analysis was conducted on 10×20 cm, TLC (Thin Layer Chromatography) plates (Merck, Silica gel 60 F_{254}) so as to determine the presence of naphthoquinones. TLC was done against authentic standards, which were isolated, purified and identified according to the published methods (Lall et al., 2005; Van der Kooy et al., 2006). The eluting system consisted of hexane: ethyl acetate (3:1). Separated components were visualized under visible and two ultraviolet light wavelengths (254 nm and 366 nm). Thereafter the TLC plates were then sprayed with vanillin reagent for further resolution of compounds in each sample.

All the chloroform extracts were quantitatively analyzed by means of an HPLC equipped with diode array detector UV6000LP and a Phenomenex Luna column (C18 (**2**) 3 μm, 150×4.6 mm). The mobile phase consisted of acetonitrile (MeCN): water (H_2O): acetic acid (HAc) in the ratio (62.5: 32.5: 0.5) and was used in isocratic mode at a flow rate of 0.8 mL/min at 25 °C. The run time for each injection was 22 min. Each crude extract was dissolved in 2 ml acetonitrile, the sample injection volume was 10 μl and three injections per replicate were conducted. Individual naphthoquinones were identified based on the retention time and UV spectrum of purified standards.

For quantitative analysis, pure compounds were dissolved in acetonitrile and a range of dilutions from 22.5 μg/ml to 2.25 μg/ml was prepared. The dilutions were injected into the HPLC at a

volume of 10 µl, in triplicates. The absorbance wavelengths of pure 7-methyljuglone and diospyrin were 430 nm, whereas shinanolone was detected at 325 nm. Each sample extract was injected four times and their particular quantities were determined by standard curves generated for each compound. Mass of ground material (g), the standard curves slopes, volume of injection and areas of individual peaks were used to calculate the concentration of each naphthoquinone in mg/kg of plant material.

2.3. Statistical analysis

The mean values of four concentrations of each secondary compound from each sample were considered for analysis. The results were statistically analysed using one-way analysis of variance (ANOVA) and least significant differences ($P=0.05$) were determined according to the MSTATC computer program.

3. Results and discussion

Preliminary examination of the chloroform extracts from shoots and roots of *E. natalensis* showed little variation in the chemical profiles of the respective samples analyzed (not shown). All three naphthoquinones (Fig. 1) were detected in all extracts except the seeds from which shinanolone (**1**) was the only compound visualized. There were very few compounds present in the fingerprints of shoot extracts and the number of compounds decreased with growth. More compounds were depicted by TLC chromatograms of root extracts. The qualitative analysis was only significant in prior assessment on the presence of the specified naphthoquinones in the crude extracts and therefore could not be used for quantification of the individual metabolites.

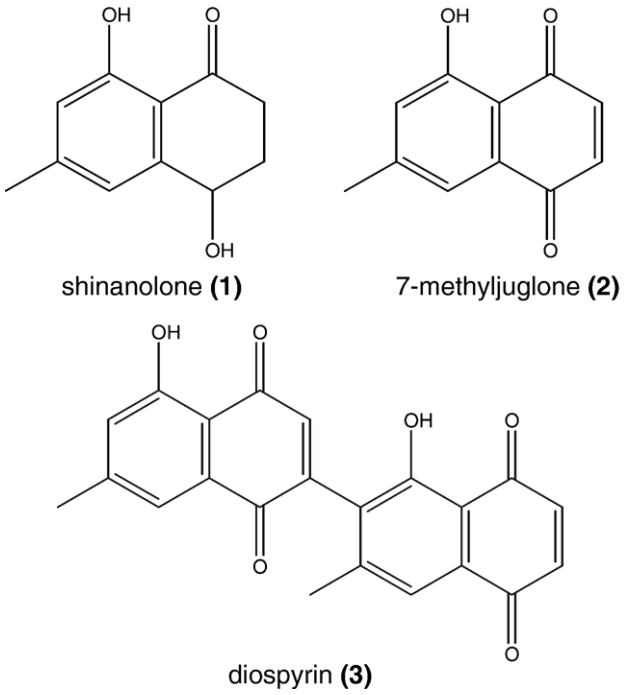

Fig. 1. Naphthoquinones quantified in seeds and seedlings of *Euclea natalensis*.

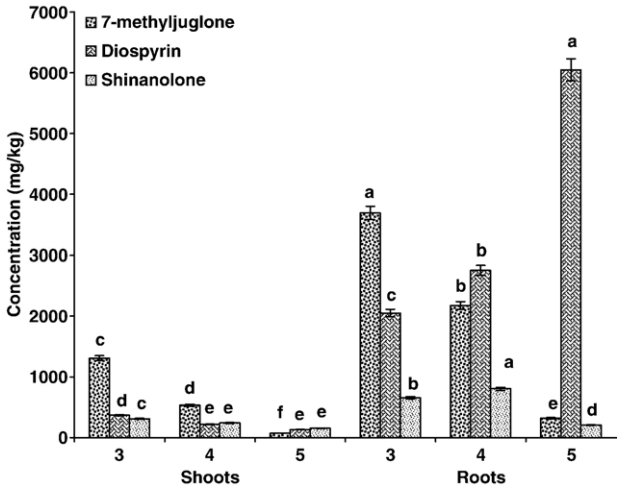

Fig. 2. Variation of naphthoquinones in shoots and roots at different stages of *Euclea natalensis* seedlings ($P<0.01$). Each value of a bar is a mean of four replicates. Values of the bars within each compound not followed by the same letter are significantly different.

HPLC analysis of naphthoquinones in seed extracts revealed the presence of diospyrin (**3**), which was not detectable from the TLC plates. Levels of the three naphthoquinones were very low or less detectible at dormancy, with shinanolone (**2**) displaying relatively higher concentrations (87.5 mg/kg) than diospyrin (**3**) and 7-methyljuglone (**2**) (6.2 and 0 mg/kg respectively). This could be attributed to the fact that at dormancy, seeds are mainly protected from attack by microorganisms by dehydration and the impermeability of the seed coat (Baskin and Baskin, 1998; Ceballos et al., 1998). In general, secondary metabolites in seeds accumulate in relatively low concentrations when compared to primary products such as starch and lipids (Mayer and Poljakoff-Mayber, 1982).

The quantitative analysis of chloroform extracts from shoots clearly showed a decrease in naphthoquinone accumulation during growth (Fig. 2). The three naphthoquinones increased rapidly during the third growth stage, with an obviously elevated content of 7-methyljuglone (**2**) (1310 mg/kg), which was approximately three times higher than shinanolone (**1**) and diospyrin (**3**) (260.4 and 369.5 mg/kg respectively). The observed increase correlated with the shoot development initiation of the hypocotyls, which were partly underground and therefore required the same protection as the subterranean parts. This was followed by a decline in the content of the three naphthoquinones in the fourth and fifth growth stages. There was a significant correlation ($P<0.01$) in the interaction between growth and accumulation of naphthoquinones in shoot development of *E. natalensis* seedlings.

Comparative examination of chloroform extracts from roots showed marked quantitative differences in the mean concentrations of naphthoquinones under consideration (Fig. 2). A significant correlation was established ($P<0.01$) between root growth and the accumulation of naphthoquinones. The concentration of shinanolone (**1**) fluctuated marginally during

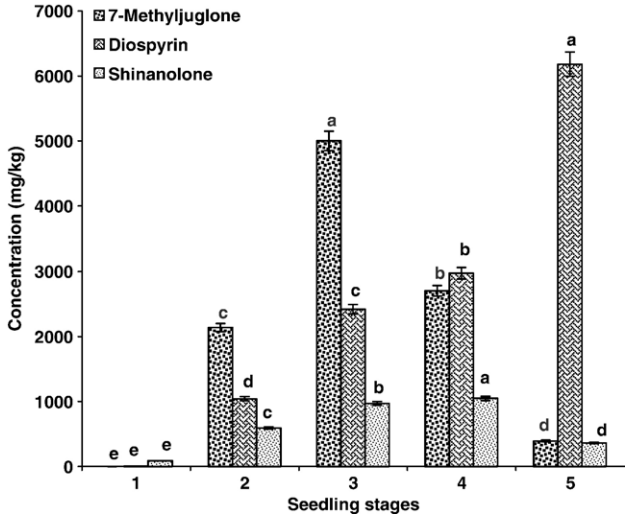

Fig. 3. Variation of naphthoquinones in different stages of *Euclea natalensis* seedling ($P<0.01$). Each value of a bar is a mean of four replicates. Values of the bars within each compound not followed by the same letter are significantly different.

root growth and its levels ranged between 208.2 and 311.5 mg/kg. The accumulation of 7-methyljuglone (**2**) peaked during the third growth stage (3693 mg/kg) and decreased significantly during the fifth growth stage (319.8 mg/kg). The mean concentrations of diospyrin (**3**) showed a different pattern to those depicted by 7-methyljuglone and shinanolone, and its accumulation was directly proportional to the relative growth of the seedlings. Diospyrin (**3**) was also detected at high levels during the fifth growth stage (6048 mg/kg), when the concentrations of the other naphthoquinones were very low. This was the highest mean value quantified from the separate shoot and root samples studied.

Considering the plant as a whole, the mean concentrations of the respective naphthoquinones studied varied significantly ($P<0.01$) during the five growth stages (Fig. 3). All the chemical constituents considered were very low at dormancy and increased progressively up to the third stage. From this stage onward, they fluctuated independently, with the exception of diospyrin (**3**), which accrued incrementally with growth. The content of shinanolone (**1**) ranged from 87.5 mg/kg to 1047 mg/kg and its high concentration was detected during the fourth seedling stage. 7-Methyljuglone (**2**) was quantified at a high level of 5003 mg/kg during the third seedling stage and was not detected from the seed samples. The minimum content of diospyrin (**3**) was detected initially at the first growth stage (6.2 mg/kg), whereas its highest mean levels were apparent during the fifth growth stage (6182 mg/kg). The level of consistency shown by the production of diospyrin (**3**) could also suggest its involvement in maximizing the fitness of seedlings. Seedlings are vulnerable to pathogen attacks and for efficient protection chemical defenses must be deployed and accumulate very early in the seedling development (Ceballos et al., 1998).

From these results it is evident that naphthoquinones are synthesized from early stages of development. The results from this study indicate that naphthoquinones accumulated mainly in the roots, with the concentrations in shoots intermediate and those in the seeds comparatively low. Repcak et al. (2000) indicated that the amount of naphthoquinones vary in different tissues of the same plant, and during the growing seasons in a given population. Based on our study, increased yields of naphthoquinones can be obtained from cultivated seedlings of *E. natalensis*. Elevated yields of shinanolone (**1**) can be obtained from the fourth seedling stage. 7-Methyljuglone (**2**) and diospyrin (**3**) could be harvested for optimum levels at the third and fifth seedling stages respectively. Large-scale production of seedlings will help in reducing the pressure that is exerted on natural population by plant gatherers.

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