

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

M.J. Bapela^{a,*}, N. Lall^a, J.H. Isaza-Martinez^{a,1}, T. Regnier^b, J.J. Marion Meyer^a

^a Department of Botany, University of Pretoria, Pretoria 0002, South Africa

^b Department of Chemistry and Physics, Tshwane University of Technology, Pretoria, South Africa

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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 detectable 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.

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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

* Corresponding author.

E-mail address: s22320688@tuks.co.za (M.J. Bapela).

¹ Present address: Grupo Polifenoles UTP, Universidad Tecnológica de Pereira, Facultad de Tecnología, Escuela de Química, La Julita A.A.079, Pereira, Risalda, Colombia.

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 shade cloth.

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 began 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 F₂₅₄) 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 (H₂O): 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 μ l, 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

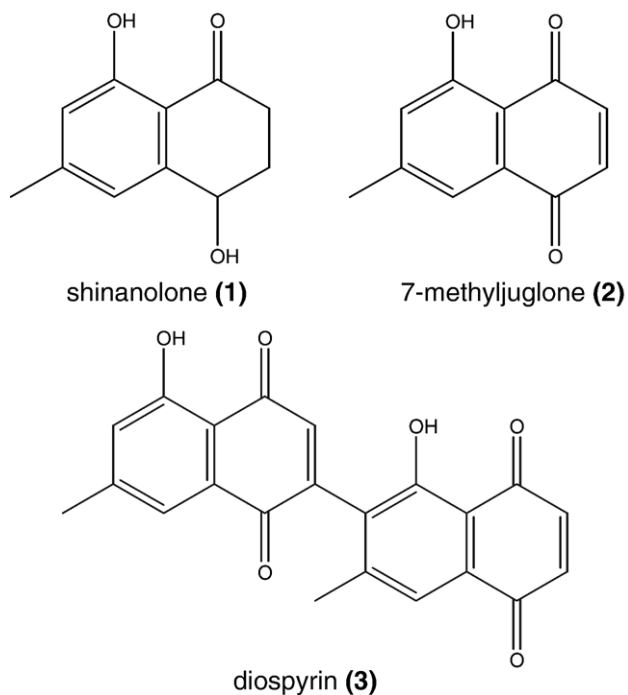


Fig. 1. Naphthoquinones quantified in seeds and seedlings of *Euclea natalensis*.

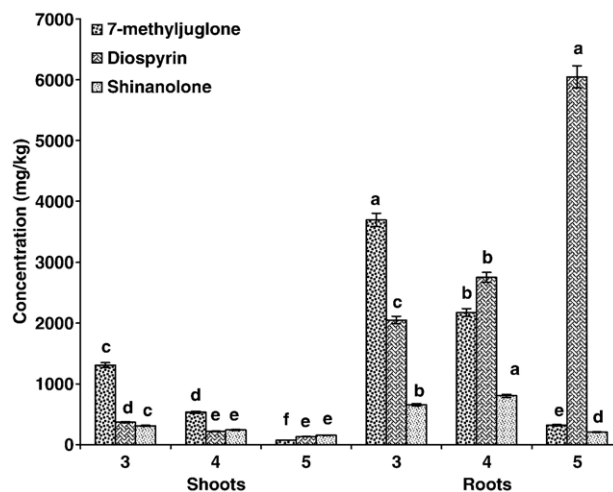


Fig. 2. Variation of naphthoquinones in shoots and roots at different stages of *Euclea natalensis* seedlings ($P < 0.01$). Each value of a bar is a mean of four replicates. Values of the bars within each compound not followed by the same letter are significantly different.

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 detectable 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

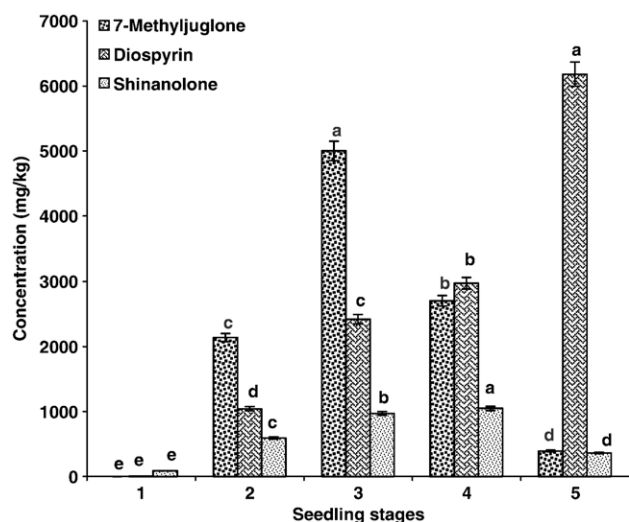


Fig. 3. Variation of naphthoquinones in different stages of *Euclea natalensis* seedling ($P < 0.01$). Each value of a bar is a mean of four replicates. Values of the bars within each compound not followed by the same letter are significantly different.

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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