South African Journal of Botany 117 (2018) 34–40

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Variations in the accumulation of three secondary metabolites in *Euclea undulata* Thunb. var. *myrtina* as a function of seasonal changes

L.E. Botha [a], G. Prinsloo [b], M.S. Deutschländer [a,*]

[a] Department of Environmental Sciences, University of South Africa, Private Bag X6, Florida 1710, South Africa
[b] Department of Agriculture and Animal Health, University of South Africa, Private Bag X6, Florida 1710, South Africa.

ARTICLE INFO

Article history:
Received 19 May 2017
Received in revised form 8 March 2018
Accepted 20 April 2018
Available online xxxx

Edited by GI Stafford

Chemical compounds studied in this article:
epicatechin (PubChem CID: 182232)
7-methyl-juglone (PubChem CID: 26905)
α-amyrin-3O-β-(5-hydroxy) ferulic acid

Keywords:
Euclea undulata
Epicatechin
α-amyrin-3O-β-(5-hydroxy) ferulic acid
7-methyl-juglone
NMR metabolomics
Seasonal environmental factors

ABSTRACT

Euclea undulata Thunb. var. *myrtina* is a widely distributed shrub in South Africa. The roots are used by traditional healers in the treatment of diabetes. Research indicated that the roots of this plant contain epicatechin and α-amyrin-3O-β-(5-hydroxy) ferulic acid, two secondary metabolites that show potential to treat diabetes. It was found that α-amyrin-3O-β-(5-hydroxy) ferulic acid inhibits α-glucosidase, while epicatechin lowers blood glucose levels. However, existing literature also indicated the presence of the naphthoquinone 7-methyl-juglone in the roots and due to its cytotoxic nature, 7-methyl-juglone poses a potential threat when *E. undulata* is used in the treatment of diabetes.

In order to assist in the effective and safe use of this plant in the treatment of diabetes, this project aimed to determine whether the presence of these metabolites is seasonal. It further aimed to contribute to more sustainable harvesting methods by investigating stems and leaves in addition to roots for the presence of these secondary metabolites.

Leaves, stems and roots were harvested from *E. undulata* plants in three different localities with different climatic conditions, with a specific focus on winter and summer rainfall regions. The plant material was air dried at room temperature and ground to a powder, which was subjected to NMR metabolomic analysis.

Multivariate data analysis resulted in clustering of samples collected in the rainy and dry seasons for both winter and summer rainfall regions, as well as delineations between roots, stems and leaves due to their secondary metabolite contents. Clustering of samples in the rainy and dry seasons of the same plants was also observed with the OPLS-DA plots indicated clustering of samples harvested during the dry and rainy seasons.

Results for the summer rainfall area indicate seasonal fluctuations in the levels of α-amyrin-3O-β-(5-hydroxy) ferulic acid in roots. It is also noteworthy that the roots always contain 7-methyl-juglone and only found in the leaves on the rainy season of the summer rainfall area. The stems can be harvested for α-amyrin-3O-β-(5-hydroxy) ferulic acid and the leaves for epicatechin in both seasons in the summer rainfall area. Results from the winter rainfall area indicate far less seasonal fluctuation. Most metabolites appear to be either present or absent in the respective organs regardless of season with the exception of epicatechin that is absent in the roots in the rainy season. Fluctuations were, however, detected in epicatechin levels in stems and 7-methyl-juglone levels in roots. From the results obtained it is evident that α-amyrin-3O-β-(5-hydroxy) ferulic acid occurs in roots, stems and leaves in the winter rainfall area and is independent of temperature whereas 7-methyl-juglone is present only in the roots. Leaves can replace the use of roots when harvested in a winter rainfall area irrespective of season as α-amyrin-3O-β-(5-hydroxy) ferulic acid and epicatechin are present and 7-methyl-juglone are absent.

1. Introduction

There has been a worldwide increase in the popularity of traditional medicine as an alternative form of healthcare. In South Africa, traditional medicine is used by roughly 70% of the population (Weideman, 2005). The interest in medicinal plants within the scientific community is driven by the fact that conventional drug treatment can result in pathogenic micro-organisms evolving and developing resistance (Gao et al., 2001).

Diabetes mellitus is a group of diseases characterised by the conditions of hyperglycaemia and glucose intolerance (Roussel, 1998).

Abbreviations: CH_3OH-d4, deuterated methanol; D_2O, deuterium water; HCA, hierarchical cluster analysis; KH_2PO_4, potassium dihydrogen phosphate; MVDA, multivariate data analysis; NMR, nuclear magnetic resonance; OPLS-DA, orthogonal partial least square discriminatory analysis; PCA, principal component analysis; TSP, trimethylsilylpropionic acid sodium salt.

* Corresponding author.
E-mail addresses: prinsg@unisa.ac.za, (G. Prinsloo), deutsms@unisa.ac.za (M.S. Deutschländer).

https://doi.org/10.1016/j.sajb.2018.04.016
0254-6299/

Motala et al. (2008) report that the occurrence of type 2 diabetes (associated with obesity) has increased in Africa over the past two decades. Statistics South Africa (2014) stated that diabetes mellitus was the fifth highest cause of natural deaths in South Africa in 2012.

The small-leaved guarri, *Euclea undulata* Thunb. var. *myrtina*, a member of the Ebenaceae family, is a common and widely distributed plant in South Africa (Van Wyk et al., 2009). Herbalists and traditional healers in South Africa use *E. undulata* for the treatment of diabetes. Assay-guided isolation of the crude acetone extract of the root bark of this plant yielded the triterpene α-amyrin-3O-β-(5-hydroxy) ferulic acid in addition to three known compounds, namely betulin, lupeol and epicatechin (Deutschländer et al., 2010). Of these compounds, α-amyrin-3O-β-(5-hydroxy) ferulic acid was found to inhibit α-glucosidase, while epicatechin lowers blood glucose levels. A potential risk associated with the use of extracts from *E. undulata* for the treatment of diabetes, is the presence of the cytotoxic naphthoquinone 7-methyl-juglone, although contradictory findings have been reported in terms of its presence (Van der Vyver and Gerritsma, 1973, 1974; Van Wyk et al., 2009; Deutschländer et al., 2010).

The influence of environmental factors on the secondary metabolism of plants has been studied extensively and is well documented in the existing literature (Gershenzon, 1984; Ianson, 2005; Tuteja and Sopory, 2008; Ghasemzadeh et al., 2010). Xu et al. (2010) describe drought stress as one of the most significant abiotic forms of stress to affect plant growth and development. Jaafar et al. (2012) identify water stress as one of the most important factors in determining the biochemical properties of plants. This is supported by the findings of this study, which showed varying levels of rainfall between seasons to coincide with fluctuations in the presence of certain secondary metabolites. This suggests that the notable differences in rainfall experienced between the summer and winter months in the areas where plants were harvested could have a significant influence on the presence of 7-methyl-juglone, epicatechin and α-amyrin-3O-β-(5-hydroxy) ferulic acid in *E. undulata*. Bapela et al., 2008 did research on the naphthoquinone content of *Euclea natalensis* roots in the Thembe National Park, KwaZulu-Natal, a summer rainfall area and found the highest 7-methyl-juglone content in the roots of trees during winter months. The results also indicated elevated levels of 7-methyl-juglone in individuals although not reflected in the mean values recorded and the apparent uniformity exhibited. In studies done on the concentrations of 7-methyl-juglone clones of *Drosera rotundifolia* populations in Finland it was found to peak in summer and spring. The results also indicated that 7-methyl-juglone concentrations varied between years and are significant between plants growing in different habitats, sand pits and bog areas. Variations in different populations were however low (Kämärainen et al., 2003).

It is hypothesised that the presence of the naphthoquinone 7-methyl-juglone, epicatechin and α-amyrin-3O-β-(5-hydroxy) ferulic acid in *E. undulata* is influenced by environmental factors that are either seasonal or non-seasonal, the latter not investigated in this study. This study investigated the noted discrepancies in the presence of 7-methyl-juglone in *E. undulata* and compared this with the presence of the key metabolites effective in the control of blood glucose levels (Van der Vyver and Gerritsma, 1973, 1974; Van Wyk et al., 2009; Deutschländer et al., 2010). Root harvesting is less sustainable than the harvesting of aerial parts, and in the interest of the preservation of wild populations it is therefore potentially beneficial to also investigate above-ground structures for the mentioned metabolites. Root, stem and leaf material were therefore investigated by collecting plant material from several locations within South Africa during different seasons to represent variations in environmental conditions.

2. Materials and methods

2.1. Study areas and collection of plant material

Root, stem and leaf samples were collected during summer (December 2013–2014) and winter seasons (August 2013–2014) from three localities. Two represented summer rainfall areas Mpumalanga (2 plants, voucher specimen number PRU121691–121692) and Gauteng (3 plants, voucher specimen numbers PRU121023–121024), and one represented the winter rainfall area, Northern Cape (3 plants, voucher specimen number Steyn 2116–2118). Plants were of the same populations in the various localities, multi-stemmed and between 1,5 and 2,0 m tall. A total of eight plants were tagged and the GPS coordinates recorded to ensure that the same specimens were sampled in the different seasons. Voucher specimens were authenticated and deposited at the H.G.W.J. Schweikerdt Herbarium at the University of Pretoria. Vegetation within the study area is exposed to dramatic seasonal changes in terms of temperature and rainfall between the summer and winter months.

2.2. Sample preparation, extraction method and data acquisition and sample analysis

Root, stem and leaf samples were dried at room temperature and ground to a homogeneous powder. Of each sample 50 mg was weighed out and direct extraction was performed in preparation for nuclear magnetic resonance (NMR) analysis. The sample preparation, extraction, data acquisition, analysis, data mining and processing were performed by adapting the standard method (Kim and Verpoorte, 2010; Nkomo et al., 2014).

A powdered sample of 50 mg per treatment was weighed in 2 mL Eppendorf tubes for extraction and analysis. Added to the samples was 0.75 mL of CH_3OH-d4 (without any standard) and 0.75 mL of potassium dihydrogen phosphate (KH_2PO_4), buffered in deuterium water (D_2O) (pH 6.0) containing 0.1% (w/w) TSP (trimethylsilylpropionic acid sodium salt). The samples were vortexed at room temperature for 1 min, ultrasonicated for 20 min at 30 °C and then centrifuged for 20 min using a microtube centrifugator (13,000 rpm, room temperature). The supernatant (1 mL) was transferred to a 5 mm NMR tube (Norell, Sigma-Aldrich) for NMR analysis.

NMR spectral data were obtained using a 600 MHz NMR spectrometer (Varian Inc., California, USA). The phasing and baseline corrections were conducted using MestReNova software (Mestrelab Research, MestReNova, version 10.0.2) with consistent settings for all sample spectra. The chemical shift ranges of methanol (δ 3.17–3.20 ppm) and water (δ 4.4–5.0 ppm) were excluded (Nkomo et al., 2014) and the remaining regions between 0.00 and 15.5 ppm were normalized for further analysis. NMR spectra were bucketed with an equal bin width of 0.04 ppm over a region of 0.00 to 15.5 ppm after completion of the phase and baseline corrections. The ASCII converted data sets were then imported into SIMCA (MKS Umetrics Simca, version 14.0) for multivariate data analysis. Multivariate analysis techniques were performed by unsupervised principal component analysis (PCA), orthogonal partial least square discriminatory analysis (OPLS-DA) and hierarchical cluster analysis (HCA) (Mncwangi et al., 2014). This was done using SIMCA-P software (MKS Umetrics, Simca, version 14.0) and the Pareto scaling method.

Multivariate data analysis (MVDA) was used to statistically analyse the collected plant material (Okada et al., 2010) for the presence of 7-methyl-juglone, epicatechin, and α-amyrin-3O-β-(5-hydroxy) ferulic acid. To determine the presence of these secondary metabolites, the regions of less importance (regions 3.2–3.8 ppm and 5.4–5.9) were removed to focus on the regions where the peaks of the three investigate compounds are present. ^{1}H NMR spectra of collected samples were compared with ^{1}H NMR spectra of epicatechin (Wishart et al., 2013), 7-methyl-juglone (Van der Kooy, 2007) and α-amyrin-3O-β-(5-hydroxy) ferulic acid (Deutschländer et al., 2010). Values found to be present on ^{1}H NMR spectra of harvested material indicate the presence of the associated metabolites within that plant sample.

PCA analyses were performed to provide an overview of the data and cluster the observations in the form of score scatter and loading plots. Score scatter plots from the PC analysis were constructed to