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Characterization of natural monatin isomers, a high intensity sweetener from the plant *Sclerochiton ilicifolius* from South Africa

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ABSTRACT

The objective was to establish the natural occurrence of the various isomers of monatin in extracts of *Sclerochiton ilicifolius* plant material harvested from different growing regions in South Africa. The natural occurrence of the 2*S*,4*S* isomer has been reported as well as the synthesis of the 2*R*,4*R* isomer. The 2*R*,4*R* is reported as the most intense sweetness however its natural occurrence has not been fully reported, as a result it was not possible to establish whether these isomers are indeed already present in the plant or come from racemisation during the processing of the plant. The presence of the monatin isomers 2*S*,4*S*; 2*R*,4*R* in aqueous extracts of *S. ilicifolius* root bark was demonstrated in each sample harvested at two different time points. The 2*R*,4*R*, 2*S*,4*S*, 2*R*,4*S*, and 2*S*,4*R* monatin isomers were absent in the aqueous extracts of *S. ilicifolius* stem and leaf samples, however was shown to be present in the root bark, and root core samples. This report confirms previous findings which suggested that the 2*S*,4*S* and 2*R*,4*R* monatin isomers occur naturally in *S. ilicifolius*.

1. Introduction

Monatin, (Indol-3-yl)-2-amino-4-carboxy-4-hydroxypentanoic acid (Fig. 1), is a naturally occurring high intensity sweetener isolated from the bark of the roots of *Sclerochiton ilicifolius*, a spiny-leafed hardwood shrub growing in the rocky hills of the Limpopo Province in South Africa (Vleggaar et al., 1992). The same authors also assigned the 2*S*,4*S* absolute configuration to the natural levorotatory compound based on NOE NMR experiments on a cyclic derivative and the application of the empirical Clough–Lutz–Jirgenson rule. The crystal structure of synthetic 2*R*,4*R* monatin potassium salt dehydrate was only recently determined by single crystal X-ray structure analysis (Amino et al., 2016). The relative sweetness of the 2*S*,4*S* monatin isolated from the natural plant was reported in 1992 to be 1200 to 1400 fold more intense than that of sucrose however synthetic 2*R*,4*R* monatin, was reported much later to have a more intense sweetness than the 2*S*,4*S* isomer *i.e.* up to 2700 times that of 5% sucrose (Patent No. WO2003059865 A1, 2003). Monatin is compatible with other sweeteners and forms acceptable blends for example with aspartame. Extensive *in vivo* toxicology studies for 2*R*,4*R* monatin have also been completed moving it closer towards commercialization (Brathwaite et al., 2011, 2014, 2016a, 2016b). The low concentration of monatin in the root bark has challenged scientists to develop processes to produce natural monatin in sizeable quantities for commercial use. While the synthesis of all four monatin stereo isomers has been reported, the natural occurrence of the three remaining stereo isomers was demonstrated in an extract of the plant (Bassoli et al., 2005). However these scientists have referenced that owing to the small amounts available and the impossibility of obtaining a larger sample or information about the exact origin and detailed extraction methodology, it was not possible to establish whether these isomers are indeed already present in the plant or come from some racemisation during the processing of the sample and suggested that this should be further investigated. This study focused on determining the natural occurrence of monatin stereo isomers in minimally processed extracts through appropriate, sustainable, controlled harvesting procedures, extraction and analysis.

As a member of the plant family Acanthaceae, *S. ilicifolius* has been referred to as 'Molomo monate' from the Sepedi name meaning "mouth nice" (Vleggaar et al., 1992). Based on actual herbarium specimens housed in the South African National Biodiversity Institute (SANBI) in Pretoria, information on plant availability is limited. The 'epicentre' of the distribution of *S. ilicifolius* is the Waterberg area in the Limpopo province of South Africa. The second 'main' area of

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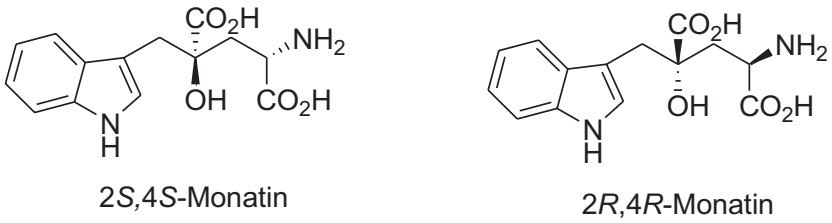

Fig. 1. 2*S*,4*S* and 2*R*, 4*R* stereoisomers of monatin.

distribution is the Zoutpansberg, a mountain range in northern Limpopo, and a third area in the Blyde River Canyon Nature Reserve near the Abel Erasmus Pass in the Mpumalanga Province.

2. Materials and methods

2.1. Chemicals and reagents

The reagents ammonium acetate, sodium bicarbonate and hydrochloric acid were purchased from Saarchem Univar, Marfey's reagent purchased from Pierce, Rockford, IL, USA, (Cat # 48895). HPLC grade acetone and acetonitrile were purchased from Burdick and Jackson (ACS/HPLC grade).

2.2. Plant material harvesting

Initial collections in September 2002 focused on the harvesting of representative samples of the root bark at each of the collection sites for the main purpose to establish the presence of the isomers, while representative portions of roots, stems, and leaves were also harvested in later collections (February 2005) to also establish the presence of monatin isomers in the other plant parts. All samples were delivered to the Council for Scientific and Industrial Research (CSIR) for further processing. Plant identification was done at the time of the collection of the research material by Hans Vahrmeijer, a registered professional Botanist (Reg. No. 400182/83 South African Council for Natural Scientific Professions). A voucher specimen was deposited in the herbarium of the SANBI (Voucher No. 00675).

2.3. Processing of plant material

All samples harvested were separately processed. For the 2002 collections, samples were immediately dried in an oven preset at 60 °C. After 48 h the samples were removed for further processing and the root samples were debarked while the root bark retained for further processing. Each sample (10–15 g) was separately ground in a high speed blender. The dried ground root bark was extracted by adding 75 ml of purified water to each of the samples. The mixture was allowed to stand at ambient temperature for 4 h and shaken manually by hand every 30 min. Each of the mixtures was separately centrifuged and the supernatant decanted. The water layer was transferred to freeze drying tubes and separately freeze dried overnight. The dried powder was transferred to pre-weighed labelled vials and stored at 4 °C prior to analysis.

For the 2005 collection, the stems, leaves samples and root samples were transferred to trays and immediately dried in an oven preset at 60 °C. After 48 h the samples were removed for further processing. The leaves were stripped and separately retained for further processing. The roots were debarked and the root bark and roots retained for further processing. Each sample was separately ground in a high speed blender. The sample was extracted by adding 200 ml of de-ionized water and the mixture was allowed to stand at ambient temperature for 4 h and shaken manually by hand every 30 min. Each of the mixtures was separately filtered through filter paper, the water layer transferred to freeze drying tubes and separately freeze dried. The dried powder was transferred to pre-weighed labelled vials and stored at 4 °C prior to analysis.

2.4. Preparation of standards

Monatin synthetic standards, 2*S*,4*S*; 2*R*,4*R* and mixture of RS/SR stereoisomers used for the characterization in the plant materials were prepared prior at the CSIR as described in literature (Rousseau et al., 2011). The standards were available at the CSIR for this research.

2.5. Chiral chromatography for separation of monatin enantiomers using Marfey's derivatization procedure (Marfey, 1984)

100 μl of a monatin standards in 10 mg/ml solution was mixed with 200 μl of 1% Marfey's reagent and 40 μl of 1 M sodium bicarbonate. The mixture was shaken and reacted at 40 °C for 1 h. After cooling to room temperature 20 μl of 2 M hydrochloric acid was added. For analysis, the derivatization mixture was diluted 100:1 (20 μl:2 ml) in water.

Approximately 15 mg of freeze-dried extract was weighed into a vial. 100 μl of water, 200 μl of 1% Marfey's reagent and then 40 μl of 1 M sodium bicarbonate was added in that order. The mixture was mixed vigorously using a vortex mixer and ultrasonication, and then reacted at 40 °C for 1 h. After cooling to room temperature 20 μl of 2 M hydrochloric acid was added. 200 μl of the derivatized mixture was diluted with 1800 μl of water and filtered for analysis.

The Chiral Method also referred to as "Marfey's", was done employing Waters Alliance 2690 HPLC, with a Quattro Micromass mass spectrometer. Compound separation was accomplished using a 250 mm × 4.6 mm reversed phase C_{18} column (Phenomenex Luna; 5 μm particle) protected by a guard column under binary gradient elution conditions (0.05% ammonium acetate/H_2O and 100% acetonitrile). The flow rate was set to 1.0 ml/Min, and the column temperature set to 40 °C. Mass spectra were collected across the *m*/*z* range of 400–

Table 1
Summary of harvested material during September 2002 and February 2005.

Ethno botanist identification	Sample ID - name/description of site	Harvest date
Sclerochiton ilicifolius	Mon I-38, Schoongelegen in Vaalwater; Limpopo province of South Africa, single plant on rocky outcrop	September 2002
Sclerochiton ilicifolius	Mon I-29, Weidehoek in Ellisras, Limpopo province of South Africa	September 2002
Sclerochiton ilicifolius	Mon I-35, Buffelshoek in Thabazimbi, Limpopo province of South Africa	September 2002
Sclerochiton ilicifolius	Mon I-32, Schoongelegen in Vaalwater, Limpopo province of South Africa,	September 2002
Sclerochiton ilicifolius	Mon I-47A1–2, Buffelshoek in Thabazimbi, Limpopo province of South Africa	February 2005
Sclerochiton ilicifolius	Mon I-48A1–2, Schoongelegen in Vaalwater, Limpopo province of South Africa	February 2005
Sclerochiton ilicifolius	Mon I-50A1–2, Weidehoek in Ellisras Limpopo province of South Africa	February 2005