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Qualitative and quantitative phytochemical characterization of *Myrothamnus flabellifolia* Welw.

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ABSTRACT

The upper aerial parts and leaf tips of *Myrothamnus flabellifolia* Welw. (Myrothamnaceae), a resurrection plant indigenous to southern Africa, are used in African traditional medicine for infections of the respiratory and urinary system as well as for inflammation of mucosa and skin. Within a phytochemical investigation of the herbal material from *M. flabellifolia* the flavonoid fraction was shown to contain quercetin **10** as well as the respective 3-*O*-β-D-galactosides, glucosides, -glucuronides and 3-*O*-α-L-rhamnosides of quercetin (**6**, **7**, **8**, **26**) and kaempferol (**1**, **2**, **3**, **9**). Additionally mono-galloylated **12**, **13**, di-galloylated **15** and tri-galloylated **16** flavonol glycosides were identified.

3,4,5-Tri-*O*-galloyl- **14** and 1,3,4,5-tetra-*O*-galloyl quinic acid **28** were isolated and characterized, beside arbutin **27** and 2″,3″-di-*O*-galloyl-arbutin **11**. Furthermore, the depside 2-*O*-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxyphenylacetic acid **5**, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose **22** and seven ellagitannins were found: 1,2-di-*O*-galloyl-(4,6-(*S*)-hexahydroxydiphenoyl)-β-D-glucose **20**, 1,3-di-*O*-galloyl-(4,6-(*S*)-hexahydroxydiphenoyl)-β-D-glucose **21**, 2,3-di-*O*-galloyl-(4,6-(*S*)-hexahydroxydiphenoyl)-β-D-glucose (tellimagrandin-I) **19**, 1,2,3-tri-*O*-galloyl-(4,6-*O*-hexahydroxydiphenoyl)-β-D-glucose (tellimagrandin-II) **23**, and two so far not described dimers of tellimagrandin-I and -II (myrodigamin-I and -II, **24**, **25**).

The presence of trehalose (3.3%), raffinose (0.2%) and stachiose (0.2%) beside a fructan (2.1%) was determined. Two ICH2-validated UHPLC methods have been developed and used within batch analysis for unambiguous identification of the herbal material and quantification of the major compounds myrodigamin-I (0.9 to 1.7%), tellimagrandin-II (0.3 to, 0.9%) and 3,4,5-tri-*O*-galloyl quinic acid (0.1 to 0.7%), besides kaempferol (0.1 to 0.3%) and quercetin content (0.2 to 0.3%) after hydrolysis of the respective glycosides.

1. Introduction

Myrothamnus flabellifolia Welw. (Myrothamnaceae) is a wooden shrub of up to 2 m height growing on the shallow and rocky soils of south and central Africa at 900 to 1200 m [1]. It is notable that only one other species of the family Myrothamnaceae, *M. moschatus*, is known which is found endemic in Madagascar [1]. To survive in the highly aride mountain environment with strong day and night temperature extremes, in connection with intense UV irradiation, and long dry winter seasons *M. flabellifolia* has developed several specialized survival techniques [2]. As a consequence the plant can turn easily into a long term desiccated quiescent state of dryness and is well known for its ability to survive long dry seasons with the ability to revitalize immediately after contact with water; *M. flabellifolia* therefore is accounted as a "resurrection plant" [3]. To avoid cellular destruction by apoptosis or necrosis during the state of dryness and for quick and effective revitalization after contact with water the plant is using typical forms of physical and chemical adaption and has also implemented histological and morphological peculiarities. Besides folding of the leaves and perpendicular arrangement of the branches during going back to a dehydrated quiescent state under preservation of chlorophyll, it comprises a lipid lining of xylem vessels [4], accumulation of the compatible solutes trehalose and sucrose as membrane and cytoplasm stabilizers [5], glycosylglycerol as osmotic protectant, the formation of volatile oil as antimicrobial defense system [6] and different galloylated hydroquinone derivatives [7] against fungal and herbivore attack. An astonishingly high content of phenolic compounds such as procyanidins [8] and hydrolysable tannins as well as flavonol derivatives may act as antioxidant protectants and natural UV blockers [9].

The upper aerial parts and leaf tips of *M. flabellifolia* Welw. are widely used within traditional African medicine for infections of the

Abbreviations: AWE, acetone-water extract of *M. flabellifolia*; CD, circular dichroism; CZE, capillary zone electrophoresis; EtOAc, ethyl acetate; FCPC, fast centrifugal partition chromatography; HHDP, hexahydroxydiphenoyl; HPLC, high performance liquid chromatography; MLCCC, multi-layer coil counter-current chromatography; LPLC, low pressure liquid chromatography; UHPLC, ultra-high pressure liquid chromatography.

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respiratory and urinary system, for wound sterilization, mastitis, halitosis, scurvy, gingivitis, and hemorrhoids [10]. Antiviral effects against *Herpes simplex* 1 [11] as well as strong antiadhesive effects against the receptor-mediated recognition and docking of *Porphyromonas gingivalis*, the main infectious cause of human periodontitis [12] have been described.

Data presented in this investigation give a close insight into the phytochemical characterization of polar and semipolar constituents of *M. flabellifolia* with the emphasis on galloylated and non-galloylated flavonols, hydrolysable tannins, quinic acid-, arbutin-derivatives, depsides and fructans.

2. Materials and methods

2.1. Plant material

Fractionation and isolation was performed on *M. flabellifolia* dried plant material originating from Northern Province, Republic of South Africa, harvested in 2006, which was obtained from Myro AG, Switzerland. This batch is named in the following as Batch 2. Identification was done by Dr. R. Plüss (Myro AG) and Dr. A. Hensel. Identification parameters were general morphology and resurrection activity (greening of the dried material with 24 to 48 h after addition of water) [13].

For quantitative analysis additionally 3 other batches of *M. flabellifolia* dried plant material were used (named in the following as Batches 1, 3 and 4), originating from Northern Province (Batch 1 and 3), and from Province Gauteng (Batch 4), Republic of South Africa.

Voucher specimens (IPBP-Myro-1 to -5) are retained in the documentation file of the Institute for Pharmaceutical Biology and Phytochemistry.

Fresh plant material from *M. flabellifolia* was obtained from the Medicinal Plant Garden of University of Münster, Germany, using a plant with the code IBS,10.06.2003 No.11 T08.08.2003, obtained from Myro AG, Switzerland and identified by AH.

2.2. General experimentation procedures

If not stated otherwise chemicals and consumables were purchased from VWR (Darmstadt, Germany), Roth (Karlsruhe, Germany) and Fluka Biochemika (Buchs, Switzerland). Solvents for HPLC were obtained in HPLC quality from Promochem (Wesel, Germany) or VWR (Darmstadt, Germany). Myricetin and myricitrin were obtained from Roth (Karlsruhe, Germany). NMR spectra were recorded in dimethylsulfoxide (purity > 99.8% δ 2.500), C_2D_6CO (purity > 99.9%, δ 2.050) and CD_3OD (purity >99.8%, δ 3.310 ppm and δ 39.520 ppm) using Varian AS 400 MHz Mercury plus, Bruker AV 400 MHz, and Varian 600 MHZ Unity Plus spectrometer.

MS data were obtained with a LCQ-Finnigan MAT, with Bruker Daltronics MicroTOF and Thermo Scientific Orbitrap LTQ XL.

CD spectra were recorded with a Jasco J-815 CD spectrometer (Jasco, Groß-Umstadt, Germany) in MeOH.

Optical rotations were measured with a Perkin-Elmer 341 digital polarimeter in MeOH.

Capillary zone electrophoresis (CZE) of the carbohydrates was performed with a P/ACE™ 50101 Beckman Coulter CE (Palo Alto, U.S.A.) with 50 mM sodium borate buffer and 4.4 M acetonitrile at pH 10.3 on a fused silica capillary with 50 μm i.d. over 77 cm. Injection 1 to 5 s, detection λ 200 nm. Derivatisation of the samples was performed according to [14].

Analytical TLC was carried out on silica gel aluminium plates (0.2 mm, VWR, Darmstadt, Germany). Identification of oligosaccharides was performed by HPAEC-PAD according the instrumentation and protocol described in [15,16].

Calibration was performed by use of α,α-trehalose (Merck, Darmstadt, Germany), D(+)-raffinose (Fluka Biochemika, Buchs, Switzerland) and stachiose (Roth, Karlsruhe, Germany) as reference compounds.

Fructan content was quantified by use of the Fructo-Oligosaccharide enzyme assay FOS (Megyzme LTD, Ireland) according to the instructions of the manufacturer; preparation of the test solution: 1.0 g dried herbal material was extracted in 80 mL water at 80 °C for 10 min; the resulting extract after centrifugation was subsequently diluted to 100 mL with water.

2.3. Extraction of the plant material and fractionation on Sephadex®LH-20

One kilogram of the dried, pulverized plant material was exhaustively extracted with cold acetone/water (7:3, 15 L, Ultra-Turrax®). The combined extracts were evaporated in vacuo, filtered to remove the precipitated chlorophyll, defatted with petroleum benzine and lyophilized to yield the crude extract AWE (188 g, 18.8%, herbal drug:extract ratio = 5.3:1). This extract was partitioned between water and EtOAc. After removal of solvents, the residues were lyophilized to yield 40 g AWE_E (4%, relate do the starting plant material) from the EtOAc phase and 139 g AWE_W (14%) from the aqueous phase.

32.6 g AWE_E, dissolved in 50 mL EtOH were fractionated by column chromatography on Sephadex® LH-20 (900 × 55 mm) using stepwise gradient elution with increasing polarity (ethanol, 28.8 L ⇒ methanol 8.74 L ⇒ acetone/water 7:3 1.5 L) to yield 23 fractions. Details on the fractions eluted at which volume, the respective masses and the respective yields are displayed as Table S1 in Supplementary data.

2.4. Chromatographic methods for fractionation of AWE_W and fractions obtained from Sephadex® LH-20

2.4.1. MPLC on MCI®-Gel

Stationary phase: MCI®-Gel CHP-20P (Mitsubishi Kasei Corp., Tokyo, Japan), 2.5 × 50 cm, Waters 510 HPLC pump, flow 4–10 mL/min, various MeOH-water gradients.

2.4.2. MPLC on RP18

Stationary phase: RP-C18, 18–32 μm, 100 Å (Besta Technik, Wilhelmsfeld, Germany), 26 × 460 cm, Waters 510 HPLC pump, flow 4–6 mL/min, various MeOH-water gradients.

2.4.3. MLCCC

Ito Multilayer Coil Separator-Extractor (P.C. Inc. Potomay, Maryland, U.S.A.), column 375 mL, i.d. 1.6 mm, injector Rheodyne 502 10.0 mL, Knauer HPLC pump 64 (Knauer, Berlin, Germany), rotation speed 800 rpm, flow 1 mL/min, solvent system: the upper phase of a chloroform:MeOH:*n*-propanol:water = 5:6:1:4 (v/v/v/v) mixture.

2.4.4. FCPC

FCPC Kromaton (Kromaton Technologies, Angers, France), injector Rheodyne 10.0 mL, Knauer HPLC pump 64 (Knauer, Berlin, Germany), rotation speed 950 resp. 1050 rpm/min, flow rate 3 mL/min resp. 10 mL/min.

Solvent system 1: the upper phase of a chloroform:MeOH:*n*-propanol:water = 5:6:1:4 (v/v/v/v) system, ascending mode.

Solvent system 2: the upper phase of a EtOAc:water = 1:1 (v/v) system.

2.4.5. Preparative HPLC

Pump: Waters 515, with Waters automated Gradient Controller (Milford, U.S.A.), detector Knauer K-2500 (Knauer, Berlin, Germany), degasser Degasys DG-2410, injection volume 1.0 mL.