Food Chemistry 133 (2012) 1373–1382

Contents lists available at SciVerse ScienceDirect

Food Chemistry

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

Isolation and structure elucidation of phenolic compounds from *Cyclopia subternata* Vogel (honeybush) intact plant and *in vitro* cultures

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ARTICLE INFO

Article history:
Received 20 October 2011
Received in revised form 19 January 2012
Accepted 27 January 2012
Available online 14 February 2012

Keywords:
Honeybush
Herbal tea
Cyclopia subternata
In vitro cultures
Benzophenones
Dihydrochalcones
Flavanones
Flavones
Isoflavones
Semi-preparative HPLC
RP-18 monolithic columns
Serially connected columns

ABSTRACT

In the presented work, an insight was made into the polyphenolic composition of intact plant material and *in vitro* cultures of indigenous South African plant *Cyclopia subternata* Vogel (honeybush). Ethyl acetate fractions of methanol extracts were separated by means of gravity column chromatography and/or semipreparative HPLC on two serially connected monolithic RP-18 columns. The structures of the isolated compounds were determined by means of 1D and 2D NMR techniques and additionally confirmed by LC-DAD-ESI-MS. Apart from the previously described honeybush components, that is mangiferin (**1**), scolymoside (**2**), hesperidin (**3**) and narirutin (**4**), three additional compounds: iriflophenone 3-*C*-*β*-glucoside (benzophenone) (**5**), phloretin 3′,5′-di-*C*-*β*-glucoside (dihydrochalcone) (**6**), and isorhoifolin (flavone) (**7**) were identified for the first time in the herb of *C. subternata*. Additionally, three isoflavone glucosides, namely calycosin 7-*O*-*β*-glucoside (**8**), rothindin (**9**) and ononin (**10**), which had not been previously reported in *Cyclopia* plants, were identified in the callus of the above species. As far as the authors are concerned, this is the first report on the presence of benzophenone and dihydrochalcone derivatives in *Cyclopia* genus.

1. Introduction

The shrubs from *Cyclopia* genus (Fabaceae family) belong to one of the most recognisable South African endemics of economic importance. Aerial parts of several *Cyclopia* species (mainly *Cyclopia intermedia* E. Mey., *Cyclopia subternata* Vogel and *Cyclopia genistoides* (L.) Vent.) are used to manufacture honeybush herbal tea, which is characterised by sweet, honey-like aroma and distinctive brown colour. The abovementioned characteristics, together with low tannin content and the lack of caffeine, clearly contribute to the growing popularity of this tisane (Joubert, Gelderblom, Louw, & De Beer, 2008). As the honeybush industry developed, research projects were undertaken in order to determine the chemical composition and the biological activities of *Cyclopia* plants. It was shown that honeybush extracts contain a rich composition of polyphenolic derivatives, including xanthones, flavanones, flavones, isoflavones and coumestans (Ferreira, Kamara, Brandt, & Joubert, 1998; Kamara, Brand, Brandt, & Joubert, 2004; Kamara, Brandt, Ferreira, & Joubert, 2003). The major compounds, which are present in all of the commercially-used *Cyclopia* plants, are two isomeric xanthone *C*-glucosides mangiferin and isomangiferin, as well as the flavanone rutinoside hesperidin (Joubert, Gelderblom et al., 2008). *Cyclopia* extracts have substantial biological effects, including antioxidative (Joubert, Gelderblom et al., 2008; Joubert et al., 2008), antimutagenic (Joubert, Gelderblom et al., 2008; Marnewick et al., 2009), anticancer (Sissing et al., 2011), phytoestrogenic (Joubert, Gelderblom et al., 2008; Mfenyana, De Beer, Joubert, & Louw, 2008; Verhoog, Joubert, & Louw, 2007), antidiabetic and cholesterol-lowering (Muller et al., 2011). Due to the growing interest towards health-promoting, anti-ageing and antioxidant-rich foods and beverages (Heinrich & Prieto, 2008), honeybush tea gradually gained international recognition, thus becoming an important export commodity. At present, *ca.* 100–150 tons of plant material are exported annually, mainly to the Netherlands, Germany, the UK, the USA and Poland. In recent years, the popularity of honeybush tea has grown remarkably fast, so the current demands exceed production capacity. As a result, efforts have been undertaken to

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doi:10.1016/j.foodchem.2012.01.114

establish *Cyclopia* plantations, in order to provide sufficient amount of material for the expanding honeybush industry.

The species of particular interest is *C. subternata*, which has been recently selected for commercial cultivation (Joubert, Joubert, Bester, De Beer, & De Lange, 2011). In comparison to other commercially-used species, e.g. *C. genistoides*, the polyphenolic fraction from *C. subternata* is characterised by a higher number of metabolites (De Beer & Joubert, 2010). The chemical composition of *C. subternata* has also been well examined and the identity of several compounds has been determined *via* isolation and subsequent NMR experiments (Kamara et al., 2004), or with the use of LC–MS (Joubert et al., 2008). However, some of the phenolic derivatives remain unidentified and only general information concerning their structure was derived from LC–DAD and LC–MS analyses (De Beer, Jerz, Joubert, Wray, & Winterhalter, 2009; De Beer & Joubert, 2010; Joubert et al., 2008).

Thus, the aim of the presented work was to isolate and identify the yet unknown constituents of *C. subternata* intact plant material. This seems essential, as the complete data concerning the chemical composition of the extract are of great value for biological activity studies. Considering that the honeybush tea is gaining popularity as an everyday, health-promoting beverage, complete recognition of its polyphenol composition is crucial. Identification of the unknown compounds can contribute to a better understanding of the antimutagenic, anticancer or oestrogenic effects of *Cyclopia* extracts (Joubert, Gelderblom et al., 2008). For instance, polyphenolic compounds which have been identified so far in the herb of *C. subternata* do not explain its phytoestrogenic activity (Mfenyana et al., 2008).

Another goal of the study was to investigate the phenolic composition of the previously obtained *C. subternata* callus, which significantly differed from the intact plant material in terms of secondary metabolite production (Kokotkiewicz, Wnuk, Bucinski, & Luczkiewicz, 2009). It was of interest, as some researchers indicate, that the induction of *in vitro* cultures of legume plants can trigger substantial changes in their production profile, including increased biosynthesis of isoflavone-type phytoallexins (Luczkiewicz & Glod, 2003). Since various branches of the phenylpropanoid pathway are active in *Cyclopia* plants (Ferreira et al., 1998; Kamara et al., 2004), it was decided to thoroughly examine *C. subternata* callus for the presence of polyphenolic compounds. Therefore, the aim of the study was to isolate the unidentified compounds from both intact plant and callus and to elucidate their structures by means of NMR spectroscopy and LC–DAD–MS. A novel approach, including the use of two serially-coupled semipreparative monolithic RP-18 columns, was applied during preparative work, which enabled fast separation of the respective analytes.

2. Materials and methods

2.1. Chemicals

The solvents used for extract preparation and column chromatography were from POCH (Gliwice, Poland). Water for preparative purposes was obtained by means of double-distillation (REL-5 double water still, POLNA, Przemysl, Poland). Acetonitrile for semi-preparative HPLC was from Merck (Darmstadt, Germany). Water for LC–MS and LC–DAD analyses was obtained with the use of DirectQ3 water purification system (Millipore, Billerica, MA), whereas acetonitrile and formic acid were from Baker (Phillipsburg, NJ). The solvents used in NMR experiments were from Deutero, Kastellaun, Germany (DMSO-d_6) and Alfa Aesar, Karlsruhe, Germany (trifluoroacetic acid-*d*). The standard substances used in LC–DAD–MS (ESI-) analyses were from Extrasynthèse, Genay, France (eriocitrin, formononetin, isorhoifolin, narirutin, ononin), Sigma–Aldrich, St. Louis, MO (mangiferin), Fluka, Buchs, Switzerland (hesperidin), Phytomarker, Tianjin, China (calycosin 7-*O*-β-D glucoside) and Chengdu Biopurify Phytochemicals, Chengdu, China (isomangiferin).

2.2. Plant materials and extracts preparation

The non-fermented ("green") *C. subternata* intact plant material was obtained from Cape Honeybush Tea (Mossel Bay, South Africa). *C. subternata* callus was previously obtained from sterile seedlings, and cultivated on modified Murashige & Skoog medium (Kokotkiewicz et al., 2009).

For extract preparation, 200-g portions of intact plant material (stems and leaves, dried after comminution, particle size <2.0 mm) and callus (freeze-dried, fine-ground, particle size <2.0 mm) were exhaustively extracted with methanol with the use of hotplate magnetic stirrer (3 × 5000 mL, 3 × 3 h, 50 °C, 200 rpm). The filtered (Filtrak 3 h paper, Bärenstein, Germany) fractions from the respective biomasses were evaporated under reduced pressure at 40 °C. The obtained dry residues were fully dissolved in 1000 mL of double-distilled water and partitioned with chloroform (3 × 1000 mL). The chloroform fractions were discarded and the remaining chloroform was removed from the water under reduced pressure (40 °C). The aqueous fractions were then partitioned with ethyl acetate (30 × 1000 mL). The ethyl acetate fractions were combined and concentrated *in vacuo* (30 °C), yielding 13 g and 6 g of dry extract for the herb and the callus, respectively. The residues were dissolved in 20 mL (herb extract) or 10 mL (callus extract) of methanol and further referred to as fraction 1 and fraction 2. The extracts were kept at –20 °C prior to use. During the liquid–liquid fractionation with ethyl acetate, precipitates were formed in aqueous phases of extracts from both intact plant (fraction 1a, 1.2 g, white powder) and callus (fraction 2a, 1 g, white powder). Another precipitate was formed during low-temperature (–20 °C) storage of fraction 1 (fraction 1b, 0.9 g, pale yellow powder). The precipitates were collected, washed with methanol (3 × 20 mL), dried and kept at –20 °C.

2.3. Semi-preparative HPLC

The semi-preparative HPLC system used in isolation procedures consisted of two L-7100 pumps (Merck Hitachi, Darmstadt, Germany), Jetstream 2 Plus column oven (WO Industrial Electronics, Langenzersdorf, Austria), variable wavelength monitor with V7566 interface (Knauer, Berlin, Germany) and injector valve (Rheodyne, Cotati, CA). The compounds were separated on two 100 × 10 mm Chromolith RP-18e columns connected in series, equipped with 10.0 × 4.6 mm guard cartridge of the same type (Merck, Darmstadt, Germany). The flow rate was 8.0 mL/min and the UV detector was set at 280 nm. All extracts subjected to HPLC separations were previously filtered through 0.45-μm nylon filters (SUN-Sri, Rockwood, TN). Data acquisition was carried out with the use of Eurochrom 2000 software (Knauer, Berlin, Germany). The detailed information concerning mobile phase, elution type, oven temperature and injection volume is given in section 2.4 referring to the isolation of the respective compounds.

2.4. Isolation procedures

2.4.1. Compounds 1, 2, 3

Fraction 1a (two compounds) was dissolved in 16.5% (*v*/*v*) acetonitrile. Due to the relatively weak solubility of the residue, a 0.05% (*w*/*v*) solution was prepared. The mixture was separated by means of semi-preparative HPLC (mobile phase: 16.5% *v*/*v* acetonitrile; isocratic elution; oven temp. 20 °C; injection volume 5000 μL).