Journal of Chromatography A, xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Use of countercurrent chromatography during isolation of 6-hydroxyluteolin-7-*O*-β-glucoside, a major antioxidant of *Athrixia phylicoides*

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ARTICLE INFO

Article history:
Available online xxx

Keywords:
Athrixia phylicoides
Chlorogenic acids
Countercurrent chromatography
Flavonoids
Quercetagetin-7-*O*-β-glucoside
6-Hydroxyluteolin-7-*O*-β-glucoside

ABSTRACT

Athrixia phylicoides, an indigenous South African herbal tea, has potential as a source of nutraceutical antioxidant extracts. Countercurrent chromatography (CCC) was employed as part of a multi-step process to isolate one of the major antioxidant compounds in *A. phylicoides* extracts. Antioxidant activity of the extracts was comparable to commercial nutraceutical extracts from *Aspalathus linearis* and *Cyclopia* spp. in a range of assays. The extracts were tested for radical scavenging (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) di-ammonium radical cation ($ABTS^{\bullet+}$) scavenging, 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging and oxygen radical absorbance capacity (ORAC)), ferric reducing antioxidant potential (FRAP) and iron chelating activity, as well as inhibition of microsomal lipid and linoleic acid emulsion oxidation. After extraction optimisation, the antioxidant activity of the major phenolic compounds in an *A. phylicoides* extract was determined using the on-line HPLC-diode-array-$DPPH^{\bullet}$ and -$ABTS^{\bullet+}$ radical scavenging assays. Major compounds reported for the first time included chlorogenic acid, 1,3-dicaffeoylquinic acid, several hydroxycinnamic acid derivatives, including dicaffeoyl quinic acids, and an unidentified flavone–hexose. Finally, CCC was used in conjunction with liquid–liquid partitioning and semi-preparative reversed-phase HPLC to isolate 6-hydroxyluteolin-7-*O*-β-glucoside (a major antioxidant) and quercetagetin-7-*O*-β-glucoside (a minor compound present in CCC fraction containing 6-hydroxyluteolin-7-*O*-β-glucoside) from an *A. phylicoides* extract. The chemical structures of the isolated compounds were confirmed by LC high-resolution electrospray ionisation MS, as well as ^{1}H, ^{13}C and 2D NMR spectroscopy. This is the first report of the isolation of these compounds from *A. phylicoides*.

1. Introduction

The success of rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.) as herbal teas on the local and global markets led to commercial interest in another indigenous South African plant, commonly known as bush tea or Zulu tea (*Athrixia phylicoides* DC., family Asteraceae, tribe Gnaphalieae) [1]. Cultivation of *A. phylicoides* is currently under investigation [2] as harvesting from natural populations cannot be sustained. The dried leaves, fine twigs and roots of the plant are traditionally used by some tribes for medicinal purposes. However, recent surveys found that the plant is now favoured as a tea for everyday use rather than for medicinal purposes. Modern users claim improvement of circulation and heart problems, as well as "cleansing" and revitalising properties [3]. Other uses include the treatment of coughs and colds and as a gargle for throat infections and loss of voice [2]. Free radical scavenging activity has been shown for aqueous extracts, infusions and ethanolic extracts of *A. phylicoides* [4], indicating that the plant could be a potential new source of antioxidants for the food, beverage, cosmetic and/or nutraceutical markets.

With the entry of new plant products on the market, it is not only important to be able to provide information on intended use such as an antioxidant ingredient, but also information on safety of use and composition. Toxicological assessment of a hot water extract of the leaves and twigs of *A. phylicoides* in vivo in rats showed no adverse effects up to very large doses [5]. The same extract also showed no hepatotoxicity in vitro or in vivo in rats [6]. In another study, its aqueous extract showed no cytotoxicity, whereas cytotoxicity was demonstrated for the ethanol extract of the leaves and twigs [4]. Due to the link between polyphenols and antioxidant activity efforts were made to elevate the total polyphenol content of the leaves through agronomic practices [7]. In spite of this interest in its polyphenols, very little is known about the phenolic composition of *A. phylicoides*. To date three phenolic compounds

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doi:10.1016/j.chroma.2010.12.096

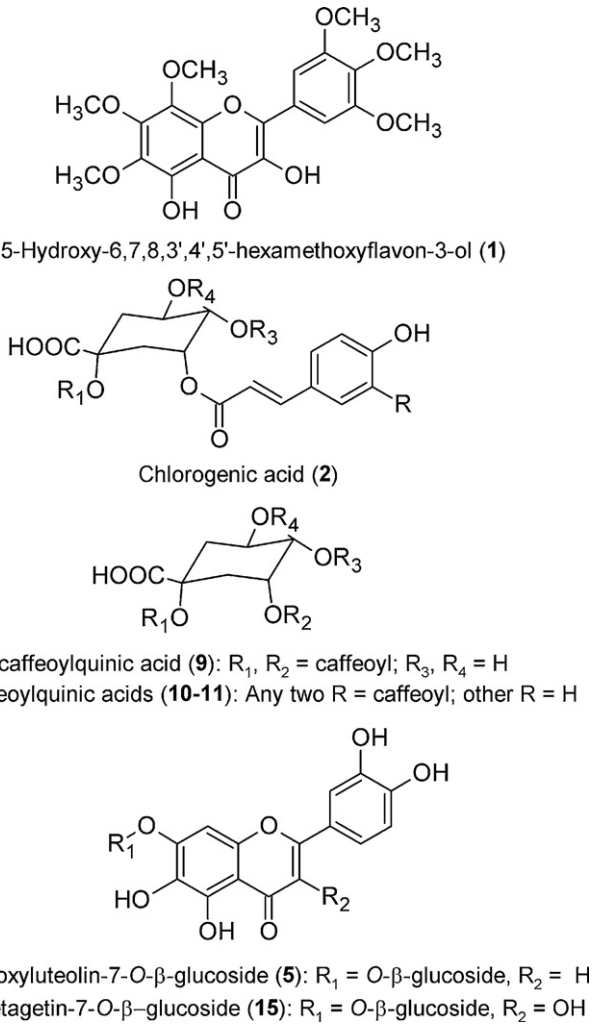

Fig. 1. Phenolic compounds in *Athrixia phylicoides*.

were isolated from *A. phylicoides*, namely *p*-hydroxyphenylpropan-3-ol coumarate [8] and its methoxy derivative [9] from the roots, and 5-hydroxy-6,7,8,3′,4′,5′-hexamethoxyflavon-3-ol (**1**) from the aerial parts (Fig. 1) [10]. Not only is its phenolic composition of interest from the perspective of antioxidant activity, but phenolic compounds also display many pharmacological activities, some of which are associated with their antioxidant activity [11]. Insight into the phenolic composition of *A. phylicoides* could help to identify specific biological targets and thus other potential medicinal uses.

The aim of this study was to characterise the antioxidant activity of *A. phylicoides* aqueous extract in a range of assays and benchmark it against commercial extracts of rooibos and honeybush. Extraction was then optimised for total phenol content and antioxidant activity in terms of extraction solvent, temperature and time. The antioxidants in the most potent extract were identified by high resolution antioxidant screening, using the on-line, post-column ABTS radical cation and DPPH radical scavenging assays, combined with high performance liquid chromatography-diode array (HPLC-DAD) analysis. This strategy is increasingly being used by natural product chemists for rapid profiling and identification of active compounds in mixtures [12]. A "semi-quantitative" approach was employed to identify the antioxidant with the highest potency relative to caffeic acid. Hydrodynamic counter-current chromatography (CCC), combined with semi-preparative HPLC, was employed to isolate and purify two flavones, namely 6-hydroxyluteolin-7-*O*-β-glucoside, a major antioxidant, and quercetagetin-7-*O*-β-glucoside, a minor constituent, for the first time from *A. phylicoides*. The identity of the isolated compounds was confirmed by liquid chromatography (LC–MS) and nuclear magnetic resonance (NMR) analysis.

2. Materials and methods

2.1. Reagents

Dimethyl sulphoxide (DMSO), DMSO-d_6, ascorbic acid, potassium persulphate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Tween 20, linoleic acid, butylated hydroxytoluene (BHT), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and gallic acid were purchased from Sigma Chemical Co. (St. Louis, USA), while 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) di-ammonium (ABTS), fluorescein disodium, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulphonic acid sodium (ferrozine), glacial acetic acid (>99.8%), caffeic acid, chlorogenic acid, protocatechuic acid and quercetin dihydrate were obtained from Fluka (Buchs, Switzerland). Acetonitrile (LiChrosolv, gradient grade for liquid chromatography), methanol (LiChrosolv, gradient grade for liquid chromatography), Folin-Ciocalteu's phenol reagent, 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) and trichloroacetic acid (TCA) were obtained from Merck (Darmstadt, Germany), while anhydrous sodium carbonate, dichloromethane, methanol, acetone and ethyl acetate were from Saarchem (Gauteng, South Africa), and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) and thiobarbituric acid (TBA) were from Aldrich (Steinheim, Germany). 1,3-Dicaffeoylquinic acid was supplied by Phytolab (Vestenbergsgreuth, Germany).

Deionised water was prepared by serial treatment of tap water with Modulab carbon, reverse-osmosis and deioniser cartridges (Continental Water Systems Corporation, San Antonio). Further purification using a Milli-Q academic water purifier (Millipore, Bedford, MA, USA) was performed to obtain HPLC grade water.

2.2. Plant material and sample preparation

A batch of air-dried *A. phylicoides* fine twigs and leaves, harvested in June 2005 in the Bushbuckridge area (Limpopo, South Africa), was identified by the South African National Botanical Institute (SANBI, Pretoria, South Africa) and supplied by Prof Jana Olivier, University of South Africa (UNISA, Pretoria, South Africa). An aqueous extract was prepared on a pilot-scale as previously described [5]. Briefly, ca. 100 L extract was prepared by boiling dried fine twigs and leaves in five batches of 1.4 kg/20 L water for 10 min. After filtration with a 125 μm mesh cloth (Polymer PES D25/35 supplied by Swiss Silk Bolting Cloth Mfg. Co. Ltd., Zurich, Switzerland), the extract was concentrated to 20 L using reverse osmosis and freeze-dried. A laboratory-scale aqueous extract was also prepared by infusing 20 g milled (Retsch type mill with 1 mm sieve, Retsch GmbH, Haan, Germany) plant material for 10 min in 200 mL freshly boiled water. The warm extract was filtered through a 125 μm mesh cloth (Polymer PES D25/35) and Whatman nr. 4 filter paper under vacuum and freeze-dried.

The aerial parts of *A. phylicoides* were also harvested in June 2007 from the Haenertzburg area (Limpopo, South Africa) and air-dried. The plant material was identified by SANBI and supplied by Prof Jana Olivier, UNISA. The plant material was milled as described and used "as-is" for water extractions. Milled plant material was defatted by exhaustive Soxhlet extraction with dichloromethane, followed by air-drying before extraction with organic solvents and water–organic solvent mixtures. Extraction yield, phenolic content and antioxidant activity (ferric reducing antioxidant potential (FRAP) and DPPH• scavenging activity) were optimised in terms of extraction solvent (experiment A: hot water, cold water, ethanol,

50% ethanol, methanol, 50% methanol, acetone, 50% acetone, ethyl acetate), temperature (experiment B: 30, 40, 50, 60, and 70 °C) and time (experiment C: 10, 20, 30, 40, 60, and 80 min) in three experiments. For experiment A, 6 g plant material was extracted using 100 mL solvent for 30 min at 30 °C, except for the hot water extraction (70 °C). All extracts were filtered through a 125 μm mesh cloth (Polymer PES D25/35) and plant material rinsed with 20 mL additional solvent before filtering through Whatman nr. 4 filter paper. In the case of hot water extracts, filtration was performed before cooling of the extract. Organic solvents were evaporated (if used) and the extracts freeze-dried. In experiment B, the same basic protocol was used and extraction performed with 50% ethanol for 30 min at different temperatures. In experiment C, the effect of extraction time was investigated at 50 °C using 50% ethanol.

Commercial extracts of indigenous South African herbal teas produced for the food and cosmetic markets included spray-dried hot water extracts of fermented rooibos (*A. linearis*) and honeybush (*Cyclopia* spp.), as well as unfermented rooibos (*A. linearis*) supplied by Afriplex (Paarl, South Africa) and flavonoid-enriched powdered extracts from unfermented rooibos, *Cyclopia subternata* and *Cyclopia genistoides* supplied by the Raps Foundation (Freising-Weihenstephan, Germany).

2.3. HPLC diode-array (DAD) analysis

An Agilent 1200 HPLC system consisting of a quaternary pump, autosampler, on-line degasser, column oven and diode-array detector (Agilent Technologies Inc., Santa Clara, CA, USA) with Chemstation 3D LC software was used for HPLC-DAD analysis. Separation was achieved on a Gemini C18 column (150 mm × 4.6 mm, 5 μm particle size) (Phenomenex, Santa Clara, CA, USA) protected by a guard column with the same stationary phase using 0.1% formic acid (A) and acetonitrile (B) at 0.8 mL/min in the following gradient: 0–30 min, 10–27% B; 30–32 min, 27% B; 32–35 min, 27–30% B; 35–45 min, 30–52% B; 45–50 min, 52–80% B; 50–55 min, 80–10% B; 55–65 min, 10% B. UV–vis spectra were recorded for all samples from 200 to 400 nm. Chromatograms were recorded at 255, 288, 320 and 350 nm. Peaks were tentatively identified by comparing retention times and UV–vis spectra from HPLC-DAD analysis with those of authentic standards. Stock solutions of chlorogenic acid, caffeic acid, 1,3-dicaffeoylquinic acid and quercetin were prepared in DMSO and aliquots frozen at −20 °C until required. Dried aqueous extracts were dissolved in deionised water prior to analysis, while the organic solvent extracts, fractions and isolated compounds were dissolved in DMSO. The standard calibration mixtures (caffeic acid, chlorogenic acid and quercetin) and sample solutions were filtered using 0.45 μm pore-size Millex-HV hydrophilic PVDF syringe filter devices (Millipore) with 4 and 33 mm diameter, respectively, prior to HPLC analysis.

2.4. LC–MS and –MS^2 analysis

LC–MS and –MS^2 analyses were performed on a Waters Acquity Ultra Performance LC (UPLC) system (Waters, Milford, MA) with quaternary pump and autosampler connected to a Waters API QTOF Ultima MS detector using electrospray ionisation in the negative mode. The column and gradient conditions were the same as for HPLC-DAD analysis. This enabled confirmation of the identity of compounds identified by comparing retention times and UV–vis spectra with those of authentic standards. Tentative identification of compounds for which no authentic standards were available was done using fragmentation pattern data from literature. The effluent from the UPLC was split and ca. 300 μL/min introduced into the MS. The LC–MS analysis parameters were as follows: desolvation temperature, 370 °C; desolvation flow rate, 370 L/h; source temperature, 100 °C; capillary voltage, 3700 V; cone gas, 50 L/h; cone voltage, 35 V. For LC–MS and –MS^2 analysis the collision energy setting was 5 and 15 arbitrary units, respectively.

2.5. Total phenol content and antioxidant activity analysis

The total phenol [13], ABTS$^{\bullet+}$ scavenging [14], DPPH$^\bullet$ scavenging [15], ferric reducing antioxidant power (FRAP) [16], oxygen radical antioxidant capacity (ORAC) [17], iron chelating [18], linoleic acid emulsion oxidation [19] and microsomal lipid peroxidation [20] assays were performed on each extract. The microsomal fraction was prepared from freshly excised male Fischer rat livers as described by van der Merwe et al. [21] and used at a final protein concentration of 1 mg/mL in the reaction volume. The total phenol content was expressed as g gallic acid equiv./100 g extract, while the antioxidant activity in the ABTS$^{\bullet+}$ scavenging, DPPH$^\bullet$ scavenging, FRAP and ORAC assays were expressed as μmol Trolox equiv./g extract. The antioxidant activity in the other assays was expressed as % inhibition for a given extract concentration. All absorbance measurements were performed on a Beckman DU-65 UV/VIS spectrophotometer (Beckman, Cape Town, South Africa) using a 1 cm path length quartz cuvette. Fluorescence measurement (ORAC assay) was performed on a Biotek Synergy HT microplate reader (Winooski, Vermont, USA).

2.6. HPLC coupled to online antioxidant assays

The optimised 50% ethanol extract (6 g milled plant material extracted for 20 min at 50 °C with 100 mL 50% ethanol) of *A. phylicoides* was subjected to online HPLC-DAD-DPPH$^\bullet$ and -ABTS$^{\bullet+}$ analyses as described by van der Merwe et al. [21]. The online system consisted of an LKB Bromma 2150 HPLC pump (Bromma, Sweden), in-line Phenomenex Degasser Model DG-4400 and LKB Bromma 2151 UV/Vis variable wavelength detector. Mixing of the HPLC effluent and the radical solution, at a flow rate of 0.5 mL/min, was achieved with a high pressure static mixing tee (Upchurch, Anatech, Cape Town, South Africa). The reaction coil was made of 15.24 m PEEK tubing (0.25 mm i.d.) to give a reaction time of ca. 0.5 min. Data were captured using DataApex Clarity v2.4.1.91 software (DataApex, Prague, Czech Republic). Scavenging of DPPH$^\bullet$ and ABTS$^{+\bullet}$ was detected as a negative peak at 515 and 430 nm, respectively. The sample was injected in duplicate at 5, 10, 15 and 20 μL. HPLC-DAD peak areas (x) at 320 nm (hydroxycinnamic acid derivatives) and 350 nm (flavones) were plotted against the corresponding negative peak areas (y) for each compound of interest. The relative antioxidant activity compared to caffeic acid was calculated by dividing the slope for the compound by the slope for caffeic acid.

2.7. Isolation of major antioxidant compound

2.7.1. Sample preparation

The optimised 50% ethanol extract was used for isolation of the major antioxidant compound. Enrichment of phenolic compounds in the extract was performed by partitioning 3.5 g extract suspended in 105 mL water with 55 mL *n*-butanol. The upper layer was removed and partitioning repeated with five 55 mL portions of *n*-butanol added to the lower layer. The pooled upper phases from each partitioning step, containing the enriched fraction (1 g), were evaporated under vacuum and freeze-dried before CCC fractionation.

2.7.2. CCC fractionation

The hydrodynamic CCC instrument used in the present study was a multilayer coil planet J-type centrifuge Spectrum model (Dynamic Extractions, Slough, UK), equipped with two preparative coils connected in series (polytetrafluoroethylene (PTFE) tubing:

1.6 mm i.d., 144 mL total volume). The inner β_r-value was measured to be 0.52 at the internal end of the coil and the outer β_r-value was 0.86 (equation $\beta_r = r/R$, in this case r is defined as the distance from the coil (planetary) axis to the nearest and farthest layer of the PTFE tubes wound on the coil system). The direction of rotation determined the *head* locations at the periphery of the two coils.

CCC fractionation was performed at 28 °C with a two-phase solvent system composed of *tert*-butyl methyl ether – acetonitrile – water (2:2:3, v/v). After thoroughly equilibrating the solvent mixtures in a separation funnel at room temperature, the two phases were separated shortly before use. The organic and aqueous phases were modified to obtain a final concentration of 600 mM formic acid and 16 mM NH_3, respectively, before degassing by ultrasonication. These modifications ensured that hydroxycinnamic acid compounds were separated from the flavone compounds due to the differences in their pK_a values. The upper organic phase was used as stationary phase and the lower aqueous phase as mobile phase in the '*head-to-tail*'-mode.

The multilayer coiled column was initially completely filled with the upper organic phase using a Gilson 305 HPLC pump (Gilson, Inc., Middleton, WI, USA) equipped with a Gilson 806 manometric module. The sample (100 mg) was dissolved in 3 mL each of upper and lower phase. The sample solution was introduced into the separation column through a manual low-pressure sample injection valve (Rheodyne, Cotati, CA, USA) and a 10 mL loop without prior column equilibration. The lower phase was pumped at a flow rate of 5 mL/min in the '*head-to-tail*' direction after start of rotation at 1600 rpm. The total run time was 40 min. The effluent stream from the *tail* outlet of the column was monitored at 320 nm using a Waters 2996 diode-array detector equipped with a semi-preparative flow cell (3 mm path length) and collected into test tubes with a Gilson FC203B fraction collector at 1.5 min intervals. After separation, the solvent in the coil was ejected with nitrogen gas to determine stationary phase retention (S_f), which was 52%. The ejected coil volume was also kept as an additional fraction. The CCC procedure was repeated eight times. The fractions from all runs were monitored using HPLC-DAD analysis and pooled into four fractions according to similarities in observed profiles. Pooled fractions were rotary evaporated under vacuum and freeze-dried (F1 = 122 mg).

2.7.3. Semi-preparative reversed-phase HPLC

Semi-preparative reversed-phase HPLC separation of F1 was performed on the Agilent 1200 series HPLC (Agilent Technologies) additionally equipped with a fraction collector. Separation took place on a Gemini C18 (150 mm × 10 mm; 5 μm particle size; 110 Å pore size) column, protected by a guard cartridge (10 mm × 10 mm) with the same stationary phase. The mobile phases, (A) 0.1% formic acid and (B) methanol, were used in the following gradient: 0–12 min, 38% B; 12–13 min, 38–60% B; 13–15 min, 60% B; 15–16 min, 60–38% B; 16–20 min, 38% B. The flow rate and column temperature were maintained at 3 mL/min and 30 °C, respectively. F1 (64 mg) was dissolved in DMSO and diluted with water (1:4) to ca. 18 mg/mL. The solution was filtered through a 0.45 μm pore-size Millex-HV hydrophilic PVDF syringe filter (Millipore) and 100 μL injected repeatedly. The fractions containing compounds **15** and **6** were collected using automatic peak detection. The pooled fractions were rotary evaporated under vacuum and freeze-dried, yielding 3.3 and 13.7 mg, respectively.

2.7.4. NMR analysis

NMR spectra was collected on a Varian ^{Unity}*Inova* 600 NMR spectrometer with a ^{1}H frequency of 600 MHz and a ^{13}C frequency of 150 MHz using a 5 mm inverse detection PFG probe. ^{1}H NMR spectra were referenced to the residual DMSO peak at 2.5 ppm and the ^{13}C spectra at 39.5 ppm. Prior to analysis samples were dissolved in DMSO-d_6 and filtered through glass wool during the transfer to the NMR tube.

3. Results and discussion

3.1. *A. phylicoides* antioxidant activity benchmarked against commercial extracts from South African herbal teas

A. phylicoides aqueous extracts from plant material obtained in 2005 were benchmarked against commercial extracts from *A. linearis* and *Cyclopia* spp. in terms of free radical scavenging activity (ABTS$^{\bullet+}$, DPPH$^\bullet$ and peroxyl radical (ORAC) scavenging assays), reducing potential (FRAP), iron chelating activity and inhibition of lipid peroxidation (microsomal lipid peroxidation and linoleic acid emulsion oxidation assays) (Table 1). These commercial extracts are currently produced for the food, nutraceutical and cosmeceutical markets and served to benchmark the aqueous extracts of *A. phylicoides*. The aspalathin-enriched extract prepared from unfermented *A. linearis* showed the highest DPPH$^\bullet$ scavenging activity, reducing potential and ability to inhibit microsomal peroxidation. On the other hand, the fermented *Cyclopia* spp. extracts were the least active in these assays. The activity of *A. phylicoides* extracts in the different assays was comparable to extracts from unfermented polyphenol-enriched *Cyclopia* spp. extracts. Results obtained for scavenging of the peroxyl radical (ORAC), a physiologically relevant radical, and the ABTS$^{\bullet+}$ were different from that obtained with DPPH$^\bullet$. Fermented *Cyclopia* spp. extracts had lower values than that of fermented *A. linearis* extracts, while unfermented polyphenol-enriched *C. subternata*, *C. genistoides* and *A. linearis* extracts had similar activity in the two assays. *A. phylicoides* aqueous extracts had ORAC and ABTS$^{\bullet+}$ scavenging values similar to that of fermented *A. linearis* extracts. *A. phylicoides* extracts showed much higher iron chelation activity than the other extracts, while all extracts demonstrated similar ability to inhibit oxidation of linoleic acid in emulsion. On the basis of these results, *A. phylicoides* is deemed to have potential

Table 1
Antioxidant activity of *Athrixia phylicoides* extracts benchmarked against commercial *Aspalathus linearis* and *Cyclopia* spp. extracts.

Extract types	Total phenol content[a]	TAC_{ABTS}[b]	TAC_{DPPH}[b]	FRAP[b]	ORAC[b]	Iron chelating ability (%)	%Inhibition of MLP	%Inhibition of LAEO
Athrixia phylicoides from 2005 plant material (n = 2)	24.16	1267	1437	873	7237	21.7	14.0	86.3
Athrixia phylicoides from 2007 plant material (n = 3)	30.57	–	1975	1418	–	–	–	–
Aspalathus linearis fermented (n = 1)	30.49	1577	2000	1382	8703	8.7	19.4	87.3
Aspalathus linearis unfermented (n = 1)	35.12	2067	2519	1764	9772	4.3	30.0	71.5
Aspalathus linearis unfermented enriched (n = 1)	42.20	3046	3149	2004	13,498	6.4	46.8	85.2
Cyclopia spp. fermented (n = 2)	17.06	977	967	661	5272	3.7	12.5	84.6
C. subternata unfermented enriched (n = 1)	25.75	1519	1681	1168	9627	3.6	24.8	87.9
C. genistoides unfermented enriched (n = 2)	24.50	1608	1366	1090	9618	5.0	22.9	85.0

[a] g gallic acid equiv./100 g.
[b] μmol Trolox equiv./g; TAC_{ABTS}, total antioxidant capacity using the ABTS$^{\bullet+}$ scavenging assay; TAC_{DPPH}, total antioxidant capacity using the DPPH$^\bullet$ scavenging assay; FRAP, ferric reducing antioxidant power; LAEO, linoleic acid emulsion oxidation; MLP, microsomal lipid peroxidation; ORAC, oxygen radical absorbance capacity.

Please cite this article in press as: D. de Beer, et al., J. Chromatogr. A (2011), doi:10.1016/j.chroma.2010.12.096

Table 2
Yield, total phenol content, antioxidant activity and compound **5** content of *Athrixia phylicoides* extracts prepared during optimisation experiments.

Extracts	Yield[a]	Total phenol content[b]	TAC_{DPPH}[c]	FRAP[c]	Compound **5**[d]
Solvent (6 g milled plant material and 100 mL solvent extracted for 30 min)					
Water (70 °C)	23.96 a[e]	30.57 b	1975 b	1418 b	0.77 d
Water (30 °C)	18.59 c	17.33 e	839 e	273 f	0.06 h
50% acetone (30 °C)	21.08 b	32.45 a	2092 a	1560 a	1.53 b
Acetone (30 °C)	0.57 g	17.61 e	854 e	797 d	0.41 ef
50% methanol (30 °C)	17.33 d	31.92 ab	2091 a	1620 a	1.37 c
Methanol (30 °C)	6.10 e	26.92 c	1575 c	1321 b	0.47 e
50% ethanol (30 °C)	17.92 cd	33.36 a	2188 a	1603 a	1.62a
Ethanol (30 °C)	1.34 f	22.70 d	1204 d	1054 c	0.37 f
Ethyl acetate (30 °C)	0.32 fg	17.92 e	852 e	571 e	0.28 g
Temperature (6 g milled plant material and 100 mL 50% ethanol extracted for 30 min)					
30 °C	17.92 c	31.14 ab	2285 a	1706 a	1.61 a
40 °C	19.50 b	30.39 b	2103 bc	1605 b	1.52 a
50 °C	20.90 a	32.42 a	2160 b	1664 a	1.60 a
60 °C	21.60 a	30.75 b	2040 c	1671 a	1.60 a
70 °C	22.01 a	30.07 b	2079 bc	1652 a	1.50 a
Time (6 g milled plant material and 100 mL 50% ethanol extracted at 50 °C)					
10 min	19.61 bi	31.45 a	2401 a	1458 ab	1.29 d
20 min	20.99 a	31.52 a	2467 a	1380 b	1.60 a
30 min	20.90 a	31.52 a	2380 a	1520 a	1.40 bcd
40 min	21.64 a	34.41 a	2412 a	1525 a	1.50 ab
60 min	21.84 a	34.72 a	2494 a	1494 ab	1.35 cd
80 min	21.90 a	32.28 a	2480 a	1466 ab	1.44 bc

TAC_{DPPH}, total antioxidant capacity using the DPPH• scavenging assay; FRAP, ferric reducing antioxidant power.
[a] g dried extract/100 g plant material extracted.
[b] g gallic acid equiv./100 g.
[c] μmol Trolox equiv./g.
[d] g quercetin equiv./100 g dried extract.
[e] Different alphabet letters for a parameter in each experiment represents significant ($P < 0.05$) differences.

for production of antioxidant-rich nutraceutical or cosmeceutical extracts.

Another batch of *A. phylicoides* plant material was obtained in 2007 from a different location. The hot water extract of this plant material gave higher total phenol, but similar antioxidant activity values (TAC_{DPPH} and FRAP) to that of fermented *A. linearis* commercial extracts (Table 1). Plant material obtained in 2007 was therefore used for extract optimisation and compound isolation.

3.2. Extraction optimisation

Extraction of phenolic antioxidants from *A. phylicoides* was optimised in terms of extraction solvent, temperature and time, as a higher phenolic content enables better sensitivity in the on-line HPLC-DAD-ABTS$^{\bullet+}$ and -DPPH$^{\bullet}$ assays due to higher signal to noise ratios. These assays were employed to identify individual compound(s) of relatively high antioxidant activity. Water and the various water–organic solvent mixtures gave higher total phenol content and antioxidant activity values than organic solvents, while the hot water extract gave the highest yield (Table 2). Extraction using 50% ethanol increased the yield with increasing extraction temperature up to 50 °C, but the total phenol content and FRAP value were not affected. Only a small decrease in TAC_{DPPH} was observed when extraction was carried out at 40 and 50 °C, compared to 30 °C. Extraction time had little effect on yield when using 50% ethanol at 50 °C. After an initial increase in yield when the extraction was prolonged from 10 to 20 min, no significant increase was observed for longer extraction times. No effect was observed for extraction time with respect to total phenol content or antioxidant activity. The final extraction conditions selected for follow-up work therefore, were 50% ethanol, 50 °C and 20 min.

3.3. Characterisation of A. phylicoides phenolic compounds

A. phylicoides optimised extract from plant material harvested in June 2007, was characterised in terms of phenolic composition using HPLC-DAD, LC–MS and $LC–MS^2$. Chlorogenic acid (**2**) and 1,3-dicaffeoylquinic acid (**9**) were identified by comparison of their retention times, UV–vis and MS characteristics with that of authentic standards. Other compounds were tentatively identified as hydroxycinnamic acid derivatives (**3**, **4**, **6**–**8**, **10**, **11**, **13**, **14**) and flavones (**5**, **12**) (Table 3).

Among the 10 hydroxycinnamic acid derivatives, multiple peaks showed pseudo-molecular ions with *m*/*z* 515 (3 peaks), 533 (5 peaks) or 695 (2 peaks). Compounds with the same pseudo-molecular ion *m*/*z* gave similar fragmentation patterns indicating that they could be structural isomers. Compounds **9**–**11** ($[M–H]^-$ = 515) correspond to dicaffeoylquinic acids, of which several structural isomers is possible [22]. This is also supported by their fragmentation pattern, namely fragment ions with *m*/*z* 353 and 191 corresponding to the consecutive loss of two caffeoyl moieties and fragment ions with *m*/*z* 191 and 173 corresponding to deprotonated quinic acid and caffeic acid fragment ions, respectively [11]. The exact identity of compounds **10** and **11** could not be ascertained using the UV–vis and MS characteristics.

The other hydroxycinnamic acid derivatives ($[M–H]^-$ = 533 and 695) seem to be related to the caffeoylquinic acid compounds and each other. Both groups gave fragment ions with *m*/*z* 371 and 209. Compounds **3**, **4** and **6**–**8** ($[M–H]^-$ = 533) gave fragment ions corresponding to the consecutive loss of two caffeoyl moieties, namely *m*/*z* 371 and 209. Fragment ions with *m*/*z* 191, corresponding to a deprotonated caffeoyl moiety, were also observed. For compounds **13** and **14** ($[M–H]^-$ = 695) the two fragment ions with *m*/*z* 371 and 209 corresponded to the loss of two and three caffeoyl moieties, respectively. Further identification of these compounds was not possible with the data at hand.

Two flavones were tentatively identified in the *A. phylicoides* optimised extract. Compound **5** represented a flavone–hexose as evidenced by the neutral loss of $\Delta m/z$ 162 resulting in a fragment ion with *m*/*z* 301. Compound **12** corresponds to a flavone-glucuronide due to the neutral loss of $\Delta m/z$ 176 resulting in a fragment ion with *m*/*z* 269.

On-line HPLC-DAD-ABTS$^{\bullet+}$ (Fig. 2) and -DPPH$^{\bullet}$ analyses of the same extract indicated that all the major peaks resulted in nega-

Table 3
UV–vis and MS characteristics of phenolic compounds in *Athrixia phylicoides* extracts and fractions. Conditions: cf. Fig. 2.

	λ_{max}	Mr	LC–MS	LC–MS^2		Identification
			Ions[a]	Precursor[a]	Product ions[a]	
2	295sh, 325	354	707, 353[b], 191	353	191[b]	Chlorogenic acid
3	295sh, 325	534	533[b], 371	533	209[b]	Hydroxycinnamic acid derivative
4	295sh, 325	534	533[b], 371	533	209[b]	Hydroxycinnamic acid derivative
5	284, 344	464	463[b]	463	463, 301[b]	Flavone–hexose
6	295sh, 325	534	533[b], 517, 371, 327	533	209[b]	Hydroxycinnamic acid derivative
7	295sh, 325	534	533[b], 517, 371, 327	533	209[b], 191	Hydroxycinnamic acid derivative
8	295sh, 325	534	533[b], 371	533	209[b], 191	Hydroxycinnamic acid derivative
9	295sh, 325	516	515[b], 353	515	353, 191[b], 179	1,3-Dicaffeoylquinic acid
10	295sh, 325	516	515[b], 353	515	353, 191[b], 179	Dicaffeoylquinic acid
11	295sh, 325	516	515[b]	515	353, 191, 179, 173[b]	Dicaffeoylquinic acid
12	266, 332	446	445[b]	445	269[b]	Flavone-glucuronide
13	295sh, 325	696	695[b]	695	371[b], 209	Hydroxycinnamic acid derivative
14	295sh, 325	696	695[b]	695	371[b], 209	Hydroxycinnamic acid derivative
15	266, 358	480	479[b]	479	479, 317[b]	Flavone–hexose

[a] *m*/*z* (relative intensity); sh, shoulder.
[b] Ion with highest intensity in spectrum.

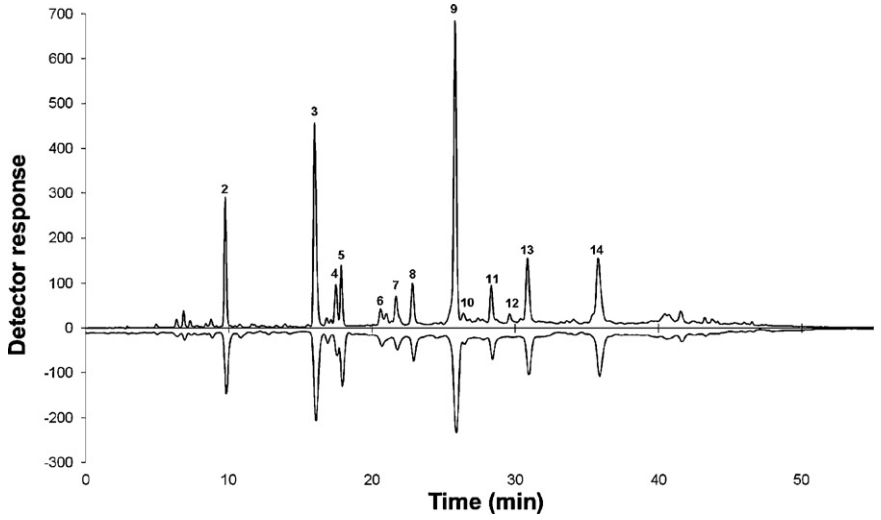

Fig. 2. HPLC-DAD (mAU) and on-line HPLC-DAD-ABTS$^{\bullet+}$ (mV) profiles of a 50% ethanol extract of *Athrixia phylicoides* from plant material harvested in Haenertzburg (June 2007). Column: gemini C18 column (150 mm × 4.6 mm i.d., 5 μm); mobile phase: acetonitrile–formic acid (0.1%) (acetonitrile: 0–30 min, 10–27%; 30–32 min, 27%; 32–35 min, 27–30%; 35–45 min, 30–52%; 45–50 min, 52–80%; 50–55 min, 80–10%; 55–65 min, 10%); flow-rate: 1.0 mL/min; detection wavelength: 320 nm. Post-column reaction: ABTS$^{\bullet+}$ reagent added at 0.5 mL/min with reaction time of 0.5 min followed by detection at 430 nm.

tive peaks due to ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ scavenging (e.g. Fig. 2). Relative antioxidant activity compared to caffeic acid was calculated using the slopes of the curves for compound concentration against negative peak area. The activity of compounds **6**, **10** and **12** could not be calculated due to partial overlap of peaks, which made peak integration impossible. Compounds **4** and **5** had high relative antioxidant activity in both assays (Fig. 3). Differences were observed between values obtained using on-line HPLC-DAD-ABTS$^{\bullet+}$ and -DPPH$^{\bullet}$ (Fig. 3). Compounds **2**–**5**, **9**, **13** and **14** gave higher relative ABTS$^{\bullet+}$ than DPPH$^{\bullet}$ radical scavenging values, while the opposite was true for compound **9**. Compounds **8** and **11** gave very similar relative ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ scavenging values. The range of relative antioxidant activity values for on-line HPLC-DAD-ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ was 0.6–1.9 and 0.6–1.7, respectively. Differences between the two assays can be ascribed to differences in the accessibility of the radical and potential steric hindrance [23] in specific molecules. Goupy et al. [23] noted that steric hindrance is a major factor influencing the initial reaction rate constant of phenolic compounds with DPPH$^{\bullet}$, e.g. much faster initial scavenging for caffeic acid compared to chlorogenic acid. The ABTS$^{\bullet+}$ is also more reactive than DPPH$^{\bullet}$, allowing higher sensitivity at the short reaction times used in the on-line antioxidant assays [24]. On the basis of the relative antioxidant activity results, compound **5** was targeted for isolation.

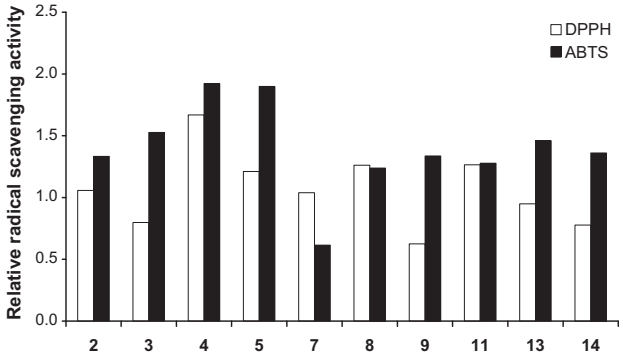

Fig. 3. Relative radical scavenging activity of *Athrixia phylicoides* phenolic compounds compared to caffeic acid.

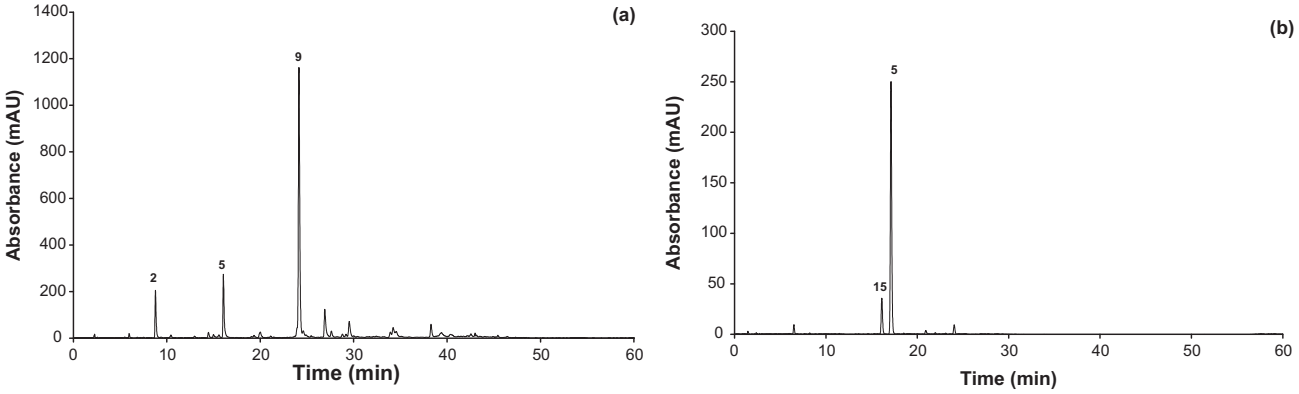

Fig. 4. HPLC-DAD profiles of *Athrixia phylicoides* enriched extract and CCC fraction 1 from plant material harvested in Haenertzburg (June 2007). Conditions: cf. Fig. 2.

3.4. Isolation and identification of antioxidant compounds in A. phylicoides

The *A. phylicoides* extract that was optimised in terms of phenolic content and antioxidant activity also represents optimal extraction of compound **5** (Table 2). However, the optimised extract is a complex mixture of compounds (Fig. 2) with only 1.6 g quercetin equiv./100 g extract of compound **5** (Table 4). The extract was, therefore, enriched using liquid–liquid partitioning of the extract in *n*-butanol–water (1:1, v/v/v). The *n*-butanol fraction was 7.2 and 4.4 times enriched in compound **5** compared to the aqueous and optimised extract, respectively. The complexity of the extract also decreased as only compounds **2**, **5** and **9** were observed as major compounds (Fig. 4a). The enriched extract was fractionated by CCC to obtain F1 (Fig. 5), which was 39.5 and 5.5 times enriched in compound **5** compared to the aqueous and *n*-butanol fractions, respectively. F1 also contained a small amount of another compound (**15**) (Fig. 4b) that was not observed in the aqueous and optimised extracts due to co-elution with compound **4**. Since CCC reduced the complexity of the extract a fairly high sample loading (ca. 1.8 mg/injection) during semi-preparative HPLC was possible, also enabling isolation of compound **15** (3.3 mg) in addition to compound **5** (13.7 mg). Compound **15**, similar to compound **5**, is a flavone–hexose as evidenced by its UV–vis and MS characteristics (Table 3). Purity of isolated **5** and **15**, measured at 245 nm using HPLC-DAD, was 94 and 91%, respectively.

NMR spectroscopy, namely ^{1}H, ^{13}C, COSY, NOESY, HSQC and HMBC experiments, was used to elucidate the structure of compound **5**. ^{1}H and ^{13}C NMR data fit exactly with those previously reported for 6-hydroxyluteolin-7-*O*-β-glucoside in literature [25,26], and its identity was confirmed by the LC–MS pseudo-molecular ion ([M+H]$^{+}$ (measured) = 465.1027 *m*/*z*; [M+H]$^{+}$ (measured) = 465.1033 *m*/*z*), LC–MS/MS spectra and the additional 2D NMR experiments performed. The MS/MS daughter ion at *m*/*z* 303 affirmed the 6-hydroxyluteolin aglycone with a neutral loss of 162 corresponding to the loss of the hexose moiety. The NOESY NMR spectra showed an n.O.e (nuclear Overhauser effect) association between the anomeric proton of the glucoside moiety and the H-8 singlet on the A-ring of the aglycone, confirming the attachment of the sugar unit to the 7-OH functional group. The large H1″ coupling constant of 6.8 Hz between H1″ and H2″ indicates a beta coupled glucose. The glycoside stereochemistry was affirmed by positive n.O.e's between H1″, 3″, 5″ and by the characteristic ^{1}H chemical shifts described in literature with the same solvent [25]. This is the first time that 6-hydroxyluteolin-7-*O*-β-glucoside has been isolated from *A. phylicoides*.

6-Hydroxyluteolin-7-*O*-β-glucoside (**5**): ^{1}H NMR (600 MHz, DMSO-d$_6$, 25 °C), δ [ppm]: 7.40 (1H, *dd*, J 2.3, 8.2 Hz, H-6′), δ 7.38 (1H, *d*, J 2.3 Hz, H-2′), δ 6.94 (s, H-8), δ 6.87 (1H, *d*, J 8.2 Hz, H-5′), δ 6.68 (1H, *s*, H-3), δ 4.99 (1H, *d*, J 6.8 Hz, H-1″), δ 3.73 (1H, *d*, J 10.3 Hz, H-6″), δ 3.18–3.52 (5H, *m*, glucose H-2″, 3″, 4″, 5″, 6″); ^{13}C NMR (600 MHz, DMSO-d$_6$, 25 °C): δ [ppm]: 182.2 (C4), δ 164.3 (C2), δ 151.3 (C7), δ 149.8 (C4′), δ 148.9 (C9), δ 146.6 (C5), δ 145.8 (C3′), δ 130.4 (C6), δ 121.6 (C1′), δ 118.9 (C6′), δ 115.9 (C5′), δ 113.5 (C2′), δ 105.8 (C10), δ 102.5 (C3), δ 100.9 (C1″), δ 93.9 (C8), δ 77.3 (C3″), δ 75.8 (C5″), δ 73.2 (C2″), δ 69.7 (C4″), δ 60.6 (C6″).

^{1}H, ^{13}C, COSY, NOESY, HSQC and HMBC experiments were also recorded for compound **15**. The ^{1}H and ^{13}C NMR spectra correspond exactly with those previously reported for quercetagetin-7-*O*-β-glucoside [25,27] and was confirmed by LC–MS pseudo-molecular ion ([M+H]$^{+}$ (measured) = 481.0964 *m*/*z*; [M+H]$^{+}$ (calculated) = 481.0982 *m*/*z*), LC–MS/MS spectra and the additional 2D NMR experiments performed. The glucoside moiety is affirmed by positive n.O.e between H1″, 3″, 5″ and by the signature ^{1}H chemical shifts described in literature in the same

Table 4
Compound **5** content (g quercetin equiv./100 g) in *Athrixia phylicoides* extracts and fractions from plant material harvested in 2007. Conditions: cf. Fig. 2.

Extract/fraction	Content	Enrichment factor[a]
Aqueous extract	0.77	–
Optimised extract	1.26	1.6
n-BuOH fraction	5.55	7.2
CCC-F1	30.42	39.5

[a] Content in extract/fraction divided by content in aqueous extract.

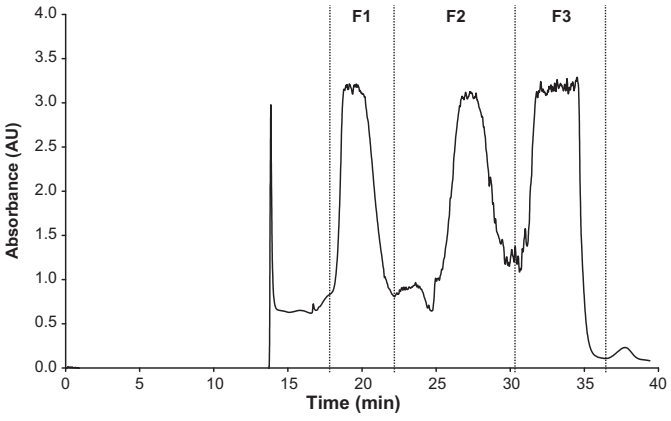

Fig. 5. CCC chromatogram for fractionation of *Athrixia phylicoides* enriched extract. Two-phase solvent system: *tert*-butyl methyl ether – acetonitrile – water (2:2:3, v/v), upper phase acidified to 600 mM formic acid and lower phase adjusted to contain 16 mM NH_3; mobile phase: the lower aqueous phase; flow rate: 5.0 mL/min; revolution speed: 1600 rpm; detection wavelength: 320 nm; sample size: 100 mg of enriched extract dissolved in 3 mL each of the lower and upper phases.

solvent [25]. The LC–MS/MS daughter ion at *m*/*z* 319 affirms the quercetagetin aglycone with a neutral loss of 162 corresponding to the loss of the hexose moiety. Here again, the large H1″ coupling constant of 7.3 Hz between H1″ and H2″ indicates a beta coupled glucoside unit. The NOESY NMR spectra showed a clear n.O.e association between the anomeric proton of the glucoside moiety and the H-8 singlet on the A-ring of the aglycone, confirming the attachment of the sugar unit to the 7-OH functional group. This is the first time that quercetagetin-7-*O*-β-glucoside has been isolated from *A. phylicoides*.

Quercetagetin-7-*O*-β-glucoside (**15**): ^{1}H NMR (600 MHz, DMSO-d_6, 25 °C), δ [ppm]: 7.67 (1H, *d*, J 2.3 Hz, H-2′), δ 7.51 (1H, *dd*, J 2.3, 8.5 Hz, H-6′), δ 6.88 (s, H-8), δ 6.86 (1H, *d*, J 8.5 Hz, H-5′), δ 4.97 (1H, *d*, J 7.3 Hz, H-1″), δ 3.72 (1H, *d*, J 10.3 Hz, H-6″), δ 3.12–3.50 (5H, *m*, H-2″, 3″, 4″, 5″, 6″); ^{13}C NMR (600 MHz, DMSO-d_6, 25 °C), δ [ppm]: 176.8 (C4), δ 152.3 (C7), δ 148.8 (C9), δ 148.5 (C4′), δ 148.2 (C2), δ 146.1 (C5), δ 145.7 (C3′), δ 136.3 (C3), δ 130.4 (C6), δ 122.7 (C1′), δ 120.6 (C6′), δ 116.2 (C5′), δ 116.1 (C2′), δ 105.8 (C10), δ 101.6 (C1″), δ 94.3 (C8), δ 77.9 (C5″), δ 76.5 (C3″), δ 73.9 (C2″), δ 70.4 (C4″), δ 61.4 (C6″).

The isolated compounds are reported for the first time from *A. phylicoides*. 6-Hydroxyluteolin-7-*O*-β-glucoside (**5**) was reported to have higher superoxide radical anion ($O_2^{\bullet -}$) scavenging activity than luteolin-7-*O*-β-glucoside due to an additional OH group on the phenolic A ring [28]. ABTS$^{\bullet +}$ and peroxyl radical scavenging activity for compound **5** was shown to be less than that of quercetin [29]. Quercetagetin-7-*O*-β-glucoside (**15**), with an additional OH group on the C-ring, should be an even stronger antioxidant than compound **5**. This is supported by Parejo et al. [27] who showed similar DPPH$^{\bullet}$, $^{\bullet}$OH and $O_2^{\bullet -}$ scavenging activity for compound **15** and quercetin. Additional OH groups, especially in the 3-position, generally increases antioxidant activity, while glycosides generally have less activity than aglycons. Compound **5** has also been reported to exhibit α-glucosidase inhibitory activity [30].

4. Conclusions

A. phylicoides displayed good potential as a new source of antioxidant-rich extracts for the nutraceutical and cosmeceutical markets, based on its antioxidant activity compared to that of commercial extracts from *A. linearis* and *Cyclopia* spp. Several major peaks with antioxidant activity were detected using on-line HPLC-DAD-ABTS$^{\bullet +}$ and -DPPH$^{\bullet}$ analyses in a "semi-quantitative" manner. CCC was used in conjunction with liquid-liquid extraction and semi-preparative HPLC to isolate 6-hydroxyluteolin-7-*O*-β-glucoside, a compound with high relative antioxidant activity, and quercetagetin-7-*O*-β-glucoside for the first time in extracts from the aerial parts of *A. phylicoides*. Hydrodynamic CCC was effective in separating the hydroxycinnamic acid derivatives in an enriched extract from the flavone constituents. Chlorogenic acid and 1,3-dicaffeoylquinic acid were also identified for the first time in *A. phylicoides* extracts by comparison of retention time, as well as UV–vis, MS and MS^2 spectra, with those of authentic standards. Several unidentified hydroxycinnamic acid derivatives were also detected. Further investigation into these compounds is needed, as they represent major peaks comprising a large proportion of the phenolic compounds present in *A. phylicoides* extracts. This study forms the basis for further investigation into the phenolic composition and bioactivity of *A. phylicoides* extracts.

Acknowledgements

Jana Olivier (University of South Africa, Pretoria, South Africa) for harvesting and supply of *Athrixia phylicoides* plant material. The National Department of Agriculture, South Africa, is gratefully acknowledged for financial support.

References

[1] E. Joubert, W.C.A. Gelderblom, A. Louw, D. de Beer, J. Ethnopharmacol. 119 (2008) 376.
[2] F.N. Mudau, H.T. Araya, E.S. du Toit, P. Soundy, J. Olivier, J. Med, Arom. Plant Sci. Biotechnol. 1 (2007) 70.
[3] I. Rampedi, J. Olivier, Acta Academica 37 (2005) 165.
[4] L.J. McGaw, V. Steenkamp, J.N. Eloff, J. Ethnopharmacol. 110 (2007) 16.
[5] N. Chellan, D. de Beer, C. Muller, E. Joubert, J. Louw, Human Exp. Toxicol. 27 (2008) 819.
[6] N. Chellan, D. de Beer, C. Muller, E. Joubert, J. Louw, 7th Congress of Toxicology in Developing Countries, September 2009, Sun City, South Africa.
[7] F.N. Mudau, P. Soundy, E.S. du Toit, J. Olivier, S. Afr. J. Bot. 72 (2006) 398.
[8] F. Bohlmann, C. Zdero, Phytochemistry 16 (1977) 1773.
[9] F. Bohlmann, M. Wallmeyer, J. Jakupovic, Phytochemistry 21 (1982) 1806.
[10] M.J. Mashimbye, F.N. Mudau, P. Soundy, T. van Ree, S. Afr. J. Chem. 59 (2006) 1.
[11] M. Clifford, J.E. Brown, in: O.M. Andersen, K.R. Markham (Eds.), Flavonoids. Chemistry, Biochemistry and Applications, Taylor & Francis, Boca Raton, FL, 2005, p. 319.
[12] S.-Y. Shi, H.-H. Zhou, Y.-P. Zhang, X.-Y. Jiang, X.-Q. Chen, K.-L. Huang, Trends Anal. Chem. 28 (2009) 865.
[13] V.L. Singleton, J.A. Rossi, Am. J. Enol. Vitic. 16 (1965) 144.
[14] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Free Radical Biol. Med. 26 (1999) 1231.
[15] A. von Gadow, E. Joubert, C.F. Hansmann, Food Chem. 60 (1997) 73.
[16] I.F.F. Benzie, J.J. Strain, Anal. Biochem. 239 (1996) 70.
[17] D. Huang, B. Ou, M. Hampsch-Woodill, J.A. Flanagan, R.L. Prior, J. Agric. Food Chem. 50 (2002) 4437.
[18] I. Gülçin, Toxicology 217 (2006) 213.
[19] H. Lingnert, K. Vallentin, C.E. Eriksson, J. Food Process. Preserv. 3 (1979) 87.
[20] D. de Beer, E. Joubert, W.C.A. Gelderblom, M. Manley, Food Chem. 90 (2005) 569.
[21] J.D. van der Merwe, E. Joubert, M. Manley, D. de Beer, C.J. Malherbe, W.C.A. Gelderblom, J. Agric. Food Chem. 58 (2010) 2214.
[22] M.N. Clifford, W. Wu, J. Kirkpatrick, N. Kuhnert, J. Agric. Food Chem. 55 (2007) 929.
[23] P. Goupy, C. Dufour, M. Loonis, O. Dangles, J. Agric. Food Chem. 51 (2003) 615.
[24] H.A.G. Niederländer, T.A. van Beek, A. Bartasiute, I.I. Koleva, J. Chromatogr. A 1210 (2008) 121.
[25] J.B. Harborne (Ed.), The Flavonoids, Advances in Research Since 1986, Chapmann & Hall, London, UK, 1994, pp. 272, 302, 446, 450, 454, 465, 468, 491.
[26] Y. Lu, L.Y. Foo, Phytochemistry 55 (2000) 263.
[27] I. Parejo, J. Bastida, F. Viladomat, C. Codina, Phytochemistry 66 (2005) 2356.
[28] Y. Lu, L.Y. Foo, Food Chem. 75 (2001) 197.
[29] F.Z. Peng, D. Strack, A. Baumert, R. Subramaniam, N.K. Goh, T.F. Chia, S.N. Tan, L.S. Chia, Phytochemistry 62 (2003) 219.
[30] J. Kawabata, K. Mizuhata, E. Sato, T. Nishioka, Y. Aoyama, T. Kasai, Phytochemistry 67 (2003) 445.