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Phytochemical characterization of South African bush tea (*Athrixia phylicoides* DC.)

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Abstract

A methanolic extract of bush tea (*Athrixia phylicoides*, Asteraceae) was evaluated sensorially. A High Temperature Liquid Chromatography (HTLC)-coupled sensory-guided analysis was performed on bush tea extract to identify potential taste modulating compounds. One fraction showed bitter enhancing effects on caffeine. Fractionation using Fast Centrifugal Partition Chromatography (FCPC) and preparative HPLC followed by structure elucidation using NMR and LC NMR led to the identification of three polymethoxylated flavones, quercetin-3′-*O*-glucoside (**1**), as well as a methoxylated derivative (**2**). In addition, two dicaffeoyl quinic acids and one coumaric acid ester (**3**) were isolated. Sensory evaluation of isolated compounds led to the identification of quercetin-3′-*O*-glucoside as bitterness enhancing principle.

Keywords: Asteracaea; *Athrixia phylicoides*; Dicaffeoylquinic acids; Fast centrifugal partition chromatography; FCPC; HTLC; Polymethoxylated flavones; Sensory-guided fractionation; Taste modulation

1. Introduction

A variety of different plants have been traditionally used as tea in South Africa. The two most prominent examples are rooibos tea (*Aspalathus linearis*) and honeybush tea (*Cyclopia* ssp.). While these two are commercially established and well known not only in Africa but also in Europe, another traditional herbal tea from South Africa, bush tea (*Athrixia phylicoides*) is still mainly used by indigenous people. *A. phylicoides*, belonging to the Asteraceae family, is a shrub from the North-Eastern mountain regions (Rampedi and Olivier, 2005). Referring to the use as herbal tea, *A. phylicoides* is locally known as bush tea, Zulu tea or Bushman's tea (Van Wyk and Gericke, 2000). Ethnobotanical use also includes medicinal purposes, such as treatment of hypertension, diabetes, heart diseases as well as gastrointestinal problems, colds and skin diseases (Joubert et al., 2008; Mudau et al., 2007; Watt and Breyer-Brandwijk, 1962). The antioxidative activity of bush tea was determined to be lower than the antioxidative capacity of commercial rooibos extracts, but higher than that of commercial honeybush extracts (Joubert et al., 2008). A report that bush tea is usually drunk with less sugar compared to other teas (Rampedi and Olivier, 2005) indicates that it might contain compounds that are able to modulate taste qualities.

The compounds described for *A. phylicoides* include different athrixianones (Bohlmann and Zdero, 1977), some phenolic acids, such as protocatechuic acid, *p*-coumaric, caffeic and chlorogenic acids, and one polymethoxylated flavonol, 5-hydroxy-6,7,8,3′,4′,5′-hexamethoxyflavon-3-ol (Mashimbye et al., 2006) were identified. De Beer at al. report the presence

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of 6-hydroxyluteolin-7-O-β-glucoside, quercetagetin-7-O-β-glucoside, 1,3-dicaffeoylquinic acid as well as two more dicaffeoylquinic acids (De Beer et al., 2011). Some of these compounds are known for their taste effects, for example, chlorogenic acid and its derivatives may contribute to the bitter taste of carrots (Kreutzmann et al., 2007), polymethoxylated flavonoids from citrus are known to contribute to the mouth feel of some citrus beverages (Kryger, 2005) and some glycosylated flavones show an astringent taste with very low thresholds (Hufnagel and Hofmann, 2008).

Therefore, in our efforts to find taste active and taste modifying compounds, a sensory-guided fractionation of the extract including a thorough phytochemical analysis was carried out using high temperature liquid chromatography (HTLC) as well as fast centrifugal partition chromatography (FCPC).

2. Material and methods

2.1. Chemicals

n-Heptane (min. 99%), ethyl acetate (p.a. > 99.5%), methanol (p.a.), ethanol (p.a. min. 99.8%), acetonitrile Chromasolv® (for HPLC, gradient grade min. 99.9%), trifluoroacetic acid (TFA), and acetic acid anhydride were purchased from Sigma–Aldrich (Steinheim, Germany). 1,2-Propanediol was obtained from Dow (Schwalbach, Germany). D_2O, CD_3OD and $CDCl_3$ were purchased from Deutero GmbH (Kastellaun, Germany).

2.2. Plant material

Leaves and twigs of *A. phylicoides* were collected near Amsterdam/Piet Retief Area in Mpumalanga/South Africa in March 2007. The material was identified by K.M. Swanepoel.

2.3. Preparation of plant extracts

Dried, ground aerial parts of *A. phylicoides* (300 g) were extracted with 2.5 L of methanol, twice at room temperature under continuous stirring for 1 h each. The extract was filtered and the filtrate evaporated *in vacuo* at 40 °C to remove residual solvent, resulting in 7.3 g of a dry green solid.

2.4. Fractionation and isolation

2.4.1. Sensory-guided fractionation via high temperature liquid chromatography (HTLC)

High temperature liquid chromatography was performed on a PRP-1 column (Hamilton, Bonaduz, Switzerland) at 120 °C (isotherm); detection was carried out with a DAD detector (λ = 385 nm) (SunChrom, Friedrichsdorf, Germany). An aliquot (0.4 g) of the crude extract (300 mg mL^{-1} ethanol/water 1:1 (*v*/*v*), injection volume 100 μL) was fractionated (F1–F18) using a H_2O/ethanol gradient (100% to 0% H_2O within 50 min) with a flow rate of 3 mL min^{-1}.

Fractions were cut peak-wise, the ethanol content was reduced below 3% *via* online-vacuum evaporation and the fractions were evaluated sensorially by a trained panel.

2.4.2. Fractionation and isolation via Fast Centrifugal Partition Chromatography (FCPC)

Fast Centrifugal Partition Chromatography (FCPC) was performed using a bench scale FCPC model, Version A (Kromaton Technologies, Angers, France) with a 200 mL semi-preparative rotor and detection on an ELSD detector SEDEX 75 (SEDERE, Alfortville Cedex, France).

Preparative HPLC was performed on a Kromasil 100-5C-18 column (5 μm, 250 × 8 mm; Eka Chemicals, Bohus, Sweden) at ambient temperature and detection with a DAD on an Ultimate 3000 system (190–800 nm) (Dionex, Idstein, Germany).

Solvent systems for FCPC fractionation of the crude extract were selected based on the ARIZONA approach (Berthod et al., 2005; Foucault and Chevolot, 1998; Pauli et al., 2008). Due to the high complexity, the crude methanolic extract (2 g) was pre-fractionated by liquid-liquid partitioning between water and organic phase as described in the literature (Kubo, 1991) with the solvent system *n*-heptane/ethyl acetate/methanol/water 2:3:2:3 (v/v) (ARIZONA mix "L") to obtain 0.46 g of a non-polar (dark green) and 1.54 g of a polar (yellow-brownish) fraction. The non-polar fraction (F_N) was again separated using *n*-heptane:ethyl acetate:methanol:water 1:1:1:1 (v/v) (ARIZONA mix "N"), while the polar fraction (F_P) was separated with a mixture of *n*-heptane:ethyl acetate:methanol:water 1:15:1:15 (v/v). For preparation of the FCPC fractionation, first the neat solvent mixtures were poured into a separation funnel at 20 °C after equilibration and the two phases were separated. FCPC fractionations were carried out on 0.5 g and 0.45 g of the polar and non-polar pre-fractionated extracts, respectively, using the methanol:water phases of the respective solvent systems as a stationary phase in the ascending mode with a flow rate of 8 mL min^{-1}. For each separation 40 fractions à 8 mL were collected; corresponding fractions were combined after LC/MS analysis. Due to impurities additional clean-up by preparative HPLC (Kromasil C-18, 250 × 8 mm; Eka Chemicals, Bohus, Sweden) using water–methanol gradients was necessary for several compounds for both analysis and sensory evaluation. 3,5-dicaffeoyl quinic acid (3 mg, F_P2), 3,4-dicaffeoyl quinic acid (2 mg, F_P2), quercetin-3′-O-glucoside (**1**, 6.5 mg, F_P3), 6-methoxyquercetin-3′-O-glucoside (**2**; 9.0 mg, F_P4), p-coumaric acid 2,6-dimethyl-6-hydroxy-oct-7-enylester (**3**; 1 mg, F_N4), 5,7,3′-hydroxy-3,6,8,4′,5′-pentamethoxy-flavone (3.0 mg, F_P1), 5,7-dihydroxy-3,6,8,3′,4′,5′-hexamethoxy flavone (9.6 mg, F_N5), and 3′-*O*-methylcalycopterin (1 mg, F_N6). The compounds were isolated as colorless to yellowish amorphous powders after evaporation of solvents *in vacuo* at 40 °C. The presence of these fractions in the crude methanolic extract is indicated in Fig. 2. FCPC fractions F_N4 (p-coumaric acid 2,6-dimethyl-6-hydroxy-oct-7-enylester), F_N6 (3′-*O*-methylcalycopterin) and F_N7 (jaceidin) were analysed *via* LC NMR in addition.

2.5. Phytochemical analysis

2.5.1. NMR

NMR spectra were recorded in CD_3OD or $CDCL_3$ on a Unity Inova (^1H: 400 MHz, ^{13}C: 100 MHz) spectrometer (Varian,

Darmstadt, Germany) at 25 °C using tetramethylsilane as an internal standard.

2.5.2. LC NMR

LC NMR measurements were carried out using D_2O (+0.01% TFA)/acetonitrile gradients analogously to adapted protocols already described in the literature (Weber et al., 2006).

2.5.3. LC/MS

LC/MS and HRMS spectra were recorded using a mass spectrometer micrOTOF-Q II (Bruker, Bremen, Germany), coupled with an Acquity UPLC system (Waters, Eschborn, Germany), equipped with a BEH C18 column (1 × 50 mm; 1.7 µm particle size; Waters, Eschborn, Germany) using a gradient of water with 0.01% formic acid and acetonitrile in ESI pos./neg. mode at a flow rate of 0.2 mL min^{-1}.

2.6. Peracetylation of 5,7-dihydroxy-3,6,8, 3′,4′,5′-hexa-methoxyflavone

Peracetylation of 5,7-dihydroxy-3,6,8,3′,4′,5′-hexamethoxy-flavone, which was isolated from the FCPC fraction of the non-polar *A. phylicoides* extract, was carried out by dissolving 8 mg of the compound in 1 mL of acetic acid anhydride, and refluxing the mixture was at 130 °C for 1 h while stirring continuously. After cooling, the residual acetic acid anhydride was removed *in vacuo* at 40 °C. The success of the peracetylation, yielding compound **4**, was checked by HR-MS prior to additional NMR experiments for the determination of the positions of the acetyl groups.

2.7. Sensory evaluation

Tasting sessions were carried out in the morning 1–2 h after breakfast, during which time the testers were asked not to drink black or green tea or coffee. An average number of 8 panelists (flavorists, expert panel) participated in each session. Samples were tested using sip and spit method. Extracts were tested at a concentration of 500 mg kg^{-1} and isolates were tested at a concentration of 100 mg kg^{-1} on testing solution. To profile the sample and to evaluate it for sweet enhancing or bitter masking properties, the sample was added onto a sucrose (5%) and caffeine solution (500 mg kg^{-1}). Flavor and taste attributes were determined by free discussion. HTLC-fractions for sensory-guided fractionation (F1-F16) were blended 1:10 with 5% sucrose and 500 ppm caffeine solution, respectively. An additional set of blank samples were prepared using deionized water, containing the same amount of ethanol as the test samples, and also blended with sucrose and caffeine solutions in the same ratio as described above. The flavor modifying effects were determined by blind duo comparison tests performed according to the protocols described earlier (Reichelt et al., 2010a). The modulating activity of the fractions is expressed in TMP (taste modulation probability) values after comparison with the corresponding blank samples.

3. Results and discussion

3.1. HTLC and subsequent sensory evaluation of A. phylicoides extract

Sensory evaluation for taste modulating effects was carried out on sucrose and caffeine solutions. The crude methanolic extract was described as tea-like and although not as bitter as for example black tea, but did not show obvious taste modulating effects. As the extract was a complex mixture of several unknown compounds, a sensory-guided fractionation using HTLC directly followed by sensory evaluation was carried out (Reichelt et al., 2010b).

A few fractions showed typical tea-like flavor attributes, e.g. bitter, astringent and herbal. In addition to these descriptors, sweet, vanilla and guiacol-like notes were detected by the testers. The fractions were subjected to a taste modulation test using a protocol described earlier (Reichelt et al., 2010a); results are shown in Fig. 1, the corresponding fraction numbers are shown in Table 1.

No fraction was found to stand out due to sweet taste enhancing or bitter reducing effects. On the contrary, several HTLC fractions, especially F2 and F7, showed high TMP values and therefore rather seemed to enhance the bitter taste of caffeine. As F7 was already described negatively (bitter, herbal, musty) during the first sensory profiling, mere additive effects might be responsible for this finding. Based on the data gained by the taste modulation test, no obvious positive taste modulating effects could be detected in the single HTLC fractions.

3.2. Characterization of crude methanolic extract

LC-MS analysis of the crude extract was carried out prior to sensory evaluation and isolation of the most interesting HTLC fractions. Re-analysis of each single fraction was carried out after fractionation by HTLC.

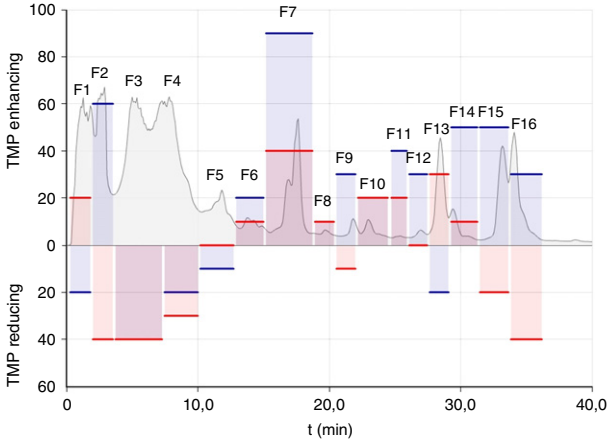

Fig. 1. Test for taste modulating effects of single fractions (F1–F16) from the methanolic *A. phylicoides* extract on 5% sucrose (red) and 500 mg kg^{-1} caffeine solution (blue) using LC Taste® (concentration 300 mg mL^{-1}; injection volume 100 µL, λ = 385 nm, n = 8. For conditions see Section 3.5.

Table 1
Sensory evaluation of HTLC fractions of *A. phylicoides* extract as shown in Fig. 1 and selected substances identified in the single fractions after re-analysis *via* LC-MS (n=2) (Reichelt et al., 2010a).

HTLC fraction	Flavor description	Compound
F1	Sour, slightly sweet, dusty	
F2	Dry, dusty, beany (weak)	Unknown (mw 354)
F3	Dry, dusty, beany (weak)	Unknown (mw 180)
F4	Tea-like, bitter (weak)	3,5-Dicaffeoyl quinic acid, 3,4-dicaffeoyl quinic acid, unknown (2x mw 538), unknown (mw 164)
F5	Bitter (weak), phenolic, fruity (weak)	Unknown (mw 432)
F6	Fruity (weak), bitter (weak), phenolic, balsamic, animalic	Unknown (mw 304)
F7	Bitter, herbal, musty, slightly sweet, balsamic, vanilla, long lasting	Quercetin-3′-O-glucoside (**1**), 6-methoxyquercetin-3′-O-glucoside (**2**)
F8	Fruity, slightly sweet, bitter (weak), phenolic	Unknown (mw 288)
F10	Fruity, slightly sweet, honey (weak), guiacol-like, smoky, animalic	Unknown (mw 316)
F11	Bitter, slightly sweet	Unknown (mw 328, 330)
F2	Tea-like, astringent	
F13	Bitter, herbal, dry	5,7,3′-Trihydroxy-3,6,8,4′,5′-pentamethoxyflavone
F14	Slightly sweet, tea-like, dry, dusty	5,7,4′-Trihydroxy-3,6,3′-trimethoxyflavone
F15	Bitter, herbal, fishy, woody	5,7-Dihydroxy-3,6,8,3′,4′,5′-hexamethoxyflavone
F16	Very bitter, very astringent, herbal, slightly sweet, fruity (raspberry seed, ionone)	5,4′-Dihydroxy-3,6,7,8,3′-pentamethoxyflavone, p-coumaric acid 2,6-dimethyl-6-hydroxy-oct-7-enylester (**3**)

A total of nine different compounds from *A. phylicoides* were isolated for detailed structure elucidation and sensory profiling using fast centrifugal partition chromatography (FCPC) followed by preparative HPLC.

3.3. Isolation of compounds from A. phylicoides methanolic extract by FCPC

FCPC of the non-polar fraction (Fig. 2a) resulted in the isolation of four different compounds (jaceidin, 3′-*O*-methylcalycopterin, 5,7-dihydroxy-3,6,8,3′,4′,5′-hexamethoxy-flavone, and *p*-coumaric acid 2,6-dimethyl-6-hydroxy-oct-7-enylester (**3**)). An additional five compounds (3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, quercetin-3′-*O*-glucoside (**1**), 6-methoxyquercetin-3′-*O*-glucoside (**2**) and 5,7,3′-trihydroxy-3,6,8,4′,5′-pentamethoxyflavone) were isolated from the polar fraction (Fig. 2b).

3.4. Elucidation of chemical structures of compounds from A. phylicoides methanolic extract by ESI-TOF-HRMS and NMR

Preparative HPLC purification of fraction F_N5 isolated by FCPC from the non-polar fraction resulted in the isolation of one compound with a molecular formula of $C_{21}H_{22}O_{10}$ on the basis of an ESI-TOF-HRMS experiment (negative ion mode), showing a $[M-H]^-$ ion peak at *m/z* 433.1140 (calculated for $[C_{21}H_{21}O_{10}]^-$, 433.1135). Although ^1H NMR data for the isolated compound were in good agreement with literature data for 5-hydroxy-6,7,8,3′,4′,5′-hexamethoxy-flavon-3-ol, described earlier from *A. phylicoides* (Mashimbye et al., 2006), the ^{13}C NMR spectrum showed a clear downfield shift of C-3. HMBC and NOESY experiments suggested the position of a methoxy group at C-3 instead of C-7. Significant NOE correlations between MeO-3 and MeO-3′/MeO-5′ as well as MeO-3 and H-2′/H-6′ were observed. To confirm the structure, a peracetylation experiment was carried out to assign the positions of the hydroxyl groups to either the A- or the C-ring. The positions of the acetyl groups were assigned to C-5 and C-7 by NOE correlations between AcO-5/MeO-6 respectively MeO-3 as well as AcO-7/MeO-6 respectively MeO-8 and therefore confirmed the suggested positions of both hydroxyl groups to the A-ring. Comparison with literature data on highly methoxylated flavones confirmed the structure to be 5,7-dihydroxy-3,6,8,3′,4′,5′-hexamethoxyflavone (Fang et al., 1985; Roitman and James, 1985).

A compound with the molecular weight of 404 was isolated from F_N6. Comparison of LC/MS and NMR data with literature led to the structure of 5,4′-dihydroxy-3,6,7,8,3′-pentamethoxyflavone (3′-*O*-methylcalycopterin) (El-Ansari et al., 1991; Roitman and James, 1985).

A flavanoid, $C_{20}H_{20}O_8$, isolated from fraction F_p1, was identified as 5,7,3′-trihydroxy-3,6,8,4′,5′-pentamethoxyflavone by comparison with literature data (Roitman and James, 1985).

As a further flavonoid compound, an aglycone with the molecular formula $C_{18}H_{16}O_8$ on the basis of an ESI-TOF-HRMS experiment (negative ion mode), showing a $[M-H]^-$ ion peak at *m/z* 359.0772 was isolated from the non-polar fraction F_N7. 1- and 2-D NMR experiments and comparison with literature data led to the molecular formula of 5,7,4′-trihydroxy-3,6,3′-trimethoxyflavon (jaceidin) (Long et al., 2003).

A compound with a nominal mass of 464 ($C_{21}H_{20}O_{12}$), which was isolated from fraction F_p3, showed the spectroscopic characteristics of glycosylated quercetin. The final position of the glucose moiety in the B-ring was confirmed by an HMBC experiment and led to the structure of quercetin-3′-*O*-glucoside (**1**) (Dellius et al., 1997; Shelyuto et al., 1977; Yang et al., 1995) (Fig. 3).

In addition to the described quercetin-glucoside, a further glycosylated compound (**2**) with the molecular formula of $C_{22}H_{22}O_{13}$ on the basis of an ESI-TOF-HRMS experiment (negative ion mode), showing a $[M-H]^-$ ion peak at *m/z* 493.0988 (calcd for $C_{22}H_{21}O_{13}$ 493.0982), was isolated from

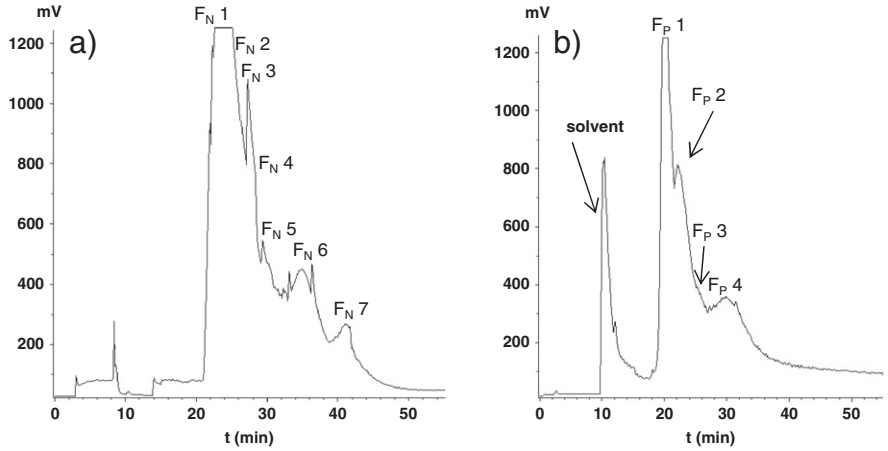

Fig. 2. FCPC chromatograms of the non-polar fraction (a) of bush tea methanolic extract (ELSD detector), using methanol/water (1:1, v/v)) as stationary phase, and *n*-heptane/ethyl acetate (1:1, v/v) as mobile phase in the ascending mode, as well as of the polar fraction (b) of bush tea methanolic extract (ELSD detector), using methanol/water (1:15, v/v) as stationary phase, and *n*-heptane/ethyl acetate (1:15, v/v) as mobile phase in the ascending mode.

F_p4. As the ^{1}H NMR spectrum showed strong similarities to the spectrum of quercetin-3′-*O*-glucoside, the same substitution pattern of ring B and the linkage of a -glucoside moiety at C-3′ was proposed. The HMBC experiment confirmed this assignment (Fig. 4).

The assumed additional methoxy group was easily detected in the ^{1}H NMR spectrum at 3.88 (s). The HMBC correlations from this MeO-6 and one aromatic proton at 6.54 (s) to the carbon at 132.2 (s, C-6) was observed. Therefore the final structure was determined to be the new compound 6-methoxyquercetin-3′-*O*-glucoside (**2**) (Fig. 3).

De Beer et al. (2011) described the presence of 1,3-dicaffeoylquinic acid as well as two other dicaffeoylquinic acids in *A. phylicoides*. After HRMS analysis of FCPC fraction F_p2 of the polar *A. phylicoides* extract, three compounds with a nominal mass were detected and the fraction subjected to additional clean-up steps. Analysis of the ^{1}H and ^{13}C NMR spectra confirmed that they were two different dicaffeoylquinic acids; the exact positions of the caffeic acid moieties were determined by comparison with published spectral data. COSY experiments were used to confirm these findings. The final structures of the molecules were in accordance with literature data of 3,5-dicaffeoylquinic acid and 3,4-dicaffeoylquinic acid. While those two compounds were isolated in sufficient amount and purity for NMR experiments, the third compound could not be isolated and the precise structure still remains unclear.

In addition to the described flavonoids and dicaffeoylquinic acids, one further compound (**3**) could be identified from non-polar fractions F_N4, which, according to UV and HRMS analyses, did not seem to be a typical flavanoid structure. This compound was determined to have the molecular formula of $C_{19}H_{26}O_4$ on the basis of its $[M\text{-}H]^-$ ion peak at *m/z* 317.1758 in its ESI-TOF-HR mass spectrum (negative ion mode). The ^{1}H NMR spectrum indicated the presence of a *p*-coumaric acid moiety as well as a *trans*-configurated olefinic double bond conjugated to the aromatic ring. The unsaturated ester moiety

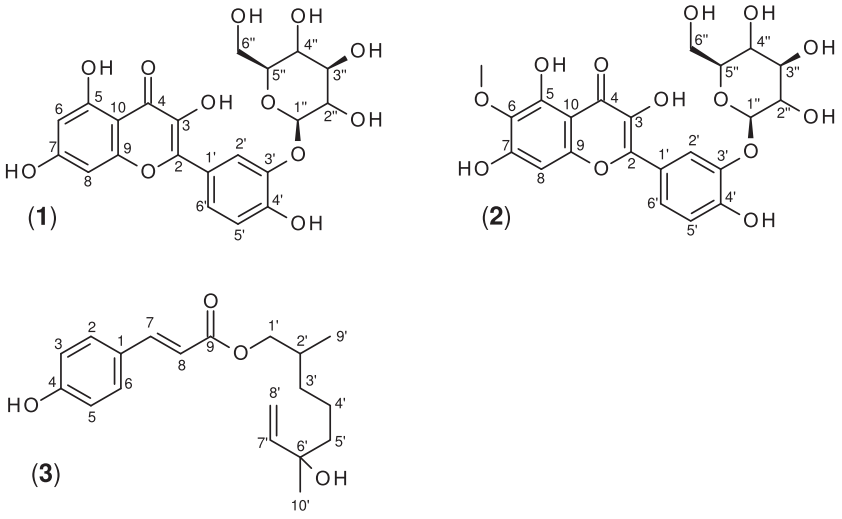

Fig. 3. Compounds **1**–**3** isolated from *Athrixia phylicoides* by Fast Centrifugal Partition Chromatography.

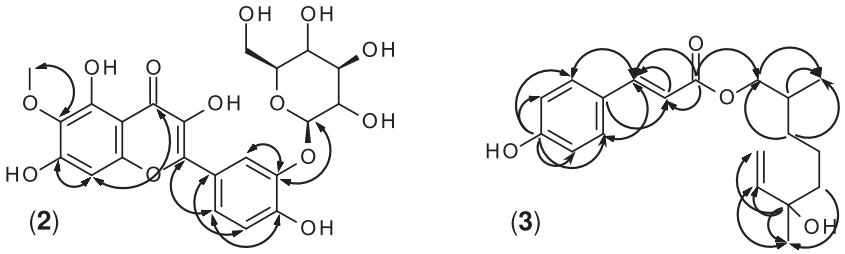

Fig. 4. gHMBC correlations for compounds **1**, **2**, **3**.

was correlated in the ^{13}C NMR spectrum with a signal at 169.2 (s) and the phenolic group in position C-4 was supported by a signal at 161.2 (s). The second part of the structure is substantiated by the signals of one methine proton at 33.6 (d, C-2′) in the ^{13}C NMR spectrum of the compound, four methylene groups at 70.1 (t, C-1′) 43.6 (t, C-5′), 35.0 (t, C-3′) and 22.3 (t, C-4′), two methyl groups at 27.4 (q, C-10′) and 17.0 (q, C-9′), one quaternary carbon atom at 73.8 (s, C-6′) and one terminal double bond at 146.4 (d, C-7′) and 111.6 (t, C-8′). HMBC correlations from C-6′ to H-8b′ at 5.05 (dd, J=1.6, 17.5 Hz), H-8a′ at 4.91 (dd, *J*=1.6, 11.0 Hz), H-7′ at 5.80 (dd, J=11.0, 17.5 Hz) and H_3-10′ (1.11, s) suggested the presence of a methylcarbinol moiety with the OH in an allylic position with respect to the terminal double bound. Furthermore, a primary alkoxy function is suggested by the ^{1}H NMR spectrum as a multiplet signal at 3.78 (m, 2H, H_2-1′) for the two disastereomeric protons which correlated with C-9′ of the second methyl group in the HMBC experiment supporting the methyl group and the methine proton to be attached to C-2′. The ester linkage of the structure is supported by an HMBC correlation of C-9 with H_2-1′. Additional NOESY and COSY correlations (Figs. 4–6) revealed compound **3** as the new *p*-coumaric acid 2,6-dimethyl-6-hydroxy-oct-7-enylester (Fig. 3).

Quercetin-3′-O-glucoside (1): ^{1}H NMR (400 MHz, D_2O/CH_3CN): δ 3.36-3.53 (4H, m, H-2″, H-3″, H-4″, H-5″), 3.67 (1H, dd, *J*=4.5 Hz, *J*=12.5 Hz, H-6a″), 3.77 (1H, d, *J*=12.5 Hz, H-6b″), 4.93 (1H, d, *J*=7.0 Hz, H-1″), 6.20 (1H, d, *J*=2.0 Hz, H-6), 6.47 (1H, d, *J*=2.0 Hz, H-8), 6.98 (1H, d, *J*=8.6 Hz, H-5′), 7.76 (1H, d, *J*=8.6 Hz, H-6′), 7.96 (1H, s, H-2′); ^{13}C NMR (100 MHz, CD_3OD): δ 62.3 (t, C-6″), 71.0 (d, C-4″), 75.9 (d, C-5″), 75.9 (d, C-2″), 77.9 (d, C-3″), 94.2 (d, C-8), 99.1 (d, C-6), 104.0 (d, C-1″), 104.5 (s, C-10), 116.7 (d, C-5′), 117.7 (d, C-2′), 124.2 (s, C-1′), 124.9 (s, C-6′), 146.7 (s, C-2, C-3′), 150.2 (s, C-4′), 158.2 (s, C-9), 165.7 (s, C-7); ESI-TOF-MS *m/z* 463 [M], 301.0354; ESI-TOF-HRMS *m/z* 463.0882 (calcd for $C_{21}H_{20}O_{12}$: 463.0877).

6-Methoxyquercetin-3′-O-glucoside (2): ^{1}H NMR (400 MHz, CD_3OD): δ 3.35-3.55 (4H, m, H-2″, H-3″, H-4″, H-5″), 3.81 (1H, *J*=dd, 4.5, 12.1Hz, H-6a″), 3.98 (1H, dd, *J*=2.4, 12.1Hz, H-6b″), 3.88 (3H, s, OCH_3-6), 4.90 (1H, d, 7.2 Hz, H-1″), 6.54 (1H, s, H-8), 6.98 (1H, d, *J*=8.7 Hz, H-5′), 7.86 (1H, d, *J*=8.5 Hz, H-6′), 8.12 (1H, s, H-2′); ^{13}C NMR (100 MHz, CD_3OD): δ 56.8 (q, OCH_3-6), 62.6 (t, C-6″), 71.4 (d, C-4″), 75.0 (d, C-2″), 76.4 (d, C-5″), 77.7 (d, C-3″), 95.1 (d, C-8), 104.4 (d, C-1″), 105.0 (s, C-10), 117.3 (d, C-5′), 118.2 (d, C-2′), 124.7 (s, C-1′), 125.1 (d, C-6′), 132.2 (s, C-6), 147.6 (s, C-2, C-3′), 150.4 (s, C-4′), 153.7 (s, C-9), 158.7 (s, C-7), 177.6 (s, C-4); ESI-TOF-MS *m/z* 493 [M], 331.0459; ESI-TOF-HRMS *m/z* 493.0988 (calcd for $C_{22}H_{22}O_{13}$: 493.0982).

p-Coumaric acid 2,6-dimethyl-6-hydroxy-oct-7-enylester (3): ^{1}H NMR (400 MHz, D_2O/CH_3CN): δ 0.85 (3H, d, *J*=6.8 Hz, H-9′), 1.05-1.42 (7H, m, H-2′, H-3′, H-4′, H-5′), 1.11 (3H, s, H-10′), 3.78 (2H, m, H-1′), 4.91 (1H, dd, *J*=1.6, 11.0 Hz, H-8a′), 5.05 (1H, dd, *J*=1.6, 17.5 Hz, H-8b′), 5.80 (1H, dd, *J*=11.0, 17.5 Hz, H-7′), 6.26 (1H, d, *J*=16.0 Hz, H-8), 6.78 (2H, d, *J*=8.4 Hz, H-3, H-5), 7.42 (2H, d, *J*=8.4 Hz, H-2, H-6), 7.53 (d, *J*=16.0 Hz, H-7); ^{13}C NMR (100 MHz, CD_3OD): 17.0 (q, C-9′), 22.3 (t, C-4′), 27.4 (q, C-10′), 33.6 (d, C-2′), 35.0 (t, C-3′), 43.6 (t, C-5′), 70.1 (t, C-1′), 73.8 (s, C-6′), 111.6 (t, C-8′), 114.9 (d, C-8), 116.5 (d, C-3, C-5), 127.0 (s, C-1), 130.8 (d, C-2), 130.8 (d, C-6), 146.1 (d, C-7), 146.4 (d, C-7′), 161.2 (s, C-4), 169,2 (s, C-9); ESI-TOF-MS *m/z* 317 [M], 145.0295; ESI-TOF-HRMS *m/z* 317.1758 (calcd for $C_{19}H_{16}O_4$: 317.1753).

5,7-Diacetoxy-3,6,8,3′,4′,5′-hexamethoxyflavone (4): ^{1}H NMR (400 MHz, CD_3OD): δ 2.43 (3H, s, OAc-7), 2.51 (3H, s, OAc-5), 3.83 (3H, s, OCH_3-3), 3.87 (3H, s, OCH_3-6), 3.93 (6H, s, OCH_3-3-, OCH_3-5′), 3.95 (3H, s, OCH_3-4′), 4.02 (3H, s, OCH_3-8), 7.45 (2H, s, H-2′, H-6′); ^{13}C NMR (100 MHz, $CDCl_3$): 20.0 (q, Ac-C5), 20.4 (q, Ac-C7), 56.2 (q, OCH_3-3′), 56.2 (q, OCH_3-5′), 60.1 (q, OCH_3-3), 61.0 (q, OCH_3-4′), 61.9 (q, OCH_3-8), 62.0 (q, OCH_3-6), 105.8 (d, C-2′, C-6′), 116.14 (s, C-10), 125.5 (s, C-1′), 137.1* (s, C-5), 139.6 (s, C-8), 140.6 (s, C-4′), 141.3 (s, C-3), 141.8* (s, C-7), 142.4 (s, C-6), 146.0* (s, C-9), 153.2 (s, C-3′, C-5′), 153.9 (s, C-2), 168.0 (s, OAc-7), 169.5 (s, OAc-5), 173.3 (s,

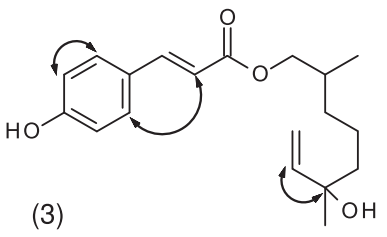

Fig. 5. Noesy correlations for compound **3**.

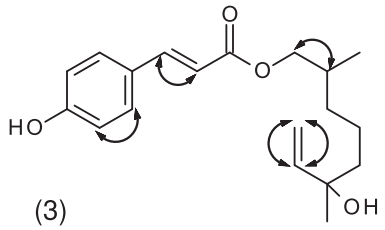

Fig. 6. gCOSY correlations for compound **3**.

C-4); ESI-TOF-MS *m/z* 519 [M], 477.1393, 435.1288; ESI-TOF-HRMS *m/z* 519.1497** (calcd for $C_{25}H_{26}O_{12}$: 519.1503).
* Interchangeable assignments.
** Positive ion mode.

3.5. Sensory evaluation of isolated Athrixia compounds

For validation of the results from the HTLC-coupled sensory analysis, the neat compounds were evaluated by sensory experiments. The isolated compounds 3′-*O*-methylcalycopterin, 5,7-dihydroxy-3,6,8,3′,4′,5′-hexamethoxyflavone, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, quercetin-3′-*O*-gluco-side (**1**), 6-methoxyquercetin-3′-*O*-glucoside (**2**) and 5,7,3′-trihydroxy -3,6,8,4′,5′-pentamethoxyflavone were judged sen-sorially in 5% sucrose solutions to detect possible sweetness modulating effects. The results of the evaluation are presented in Table 2.

Sensory evaluation of the isolated compounds confirms the results of the tests carried out by HTLC/tasting protocol: none of the compounds shows strong sensory properties. Together with a 500 ppm caffeine containing solution, fraction T7, containing quercetin-3′-*O*-glycoside (**1**) and methoxyquercetin-3′-*O*-glycoside (**2**), was perceived as more bitter by the panel than the blank caffeine solution. Sensory evaluation of isolated **1** and **2** on caffeine solution showed that the bitterness of caffeine was enhanced by the addition of **1** but not by **2**. Both compounds could not be separated under the used HTLC conditions, so that the bitter enhancing effect of the relevant fraction might also be due to combinatorial synergistic effects between both compounds. They were also evaluated at a ratio of 1:1 on caffeine to confirm this assumption. The sample was again described as bitterer than the blank caffeine sample. The sensory properties of the combined sample, however, were described to be identical to that of pure **1**. Based on the results of this sensory evaluation, it is assumed that **1** is responsible for the enhanced bitterness of the corresponding HTLC/tasting protocol fraction. Interestingly, similar results were reported for quercetin-3-*O*-glycoside in black tea (Scharbert and Hofmann, 2005). As *A. phylicoides* is reported to be caffeine-free, the effect is not found in bush tea and makes the tea less bitter than black tea. This might explain why the infusion is drunk with less sugar compared to black tea, as sugar is commonly used to mask bitterness.

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Table 2
Sensory evaluation of isolated compounds from *Athrixia phylicoides* (50 mg kg^{-1} compound on 5% sucrose solution, n=8).

Compound	Sensory description
3,4-/3,5-Dicaffeoyl quinic acids *	Slightly astringent, mouth-drying, less sweet
Quercetin-3′-O-glucoside (**1**)	Dry-dusty, slightly scratchy
6-Methoxyquercetin-3′-O-glucoside (**2**)	Slightly mouth drying, slightly bitter
5,7,3′-Trihydroxy-3,6,8,4′,5′-pentamethoxyflavone	Relatively neutral, numbing
5,7-Dihydroxy-3,6,8,3′,4′,5′-hexamethoxyflavone	Relatively neutral, tongue coating
5,4′-Dihydroxy-3,6,7,8,3′-pentamethoxyflavone	Neutral

* 1 mixture of 3,5- and 3,4-dicaffeoyl quinic acid.

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