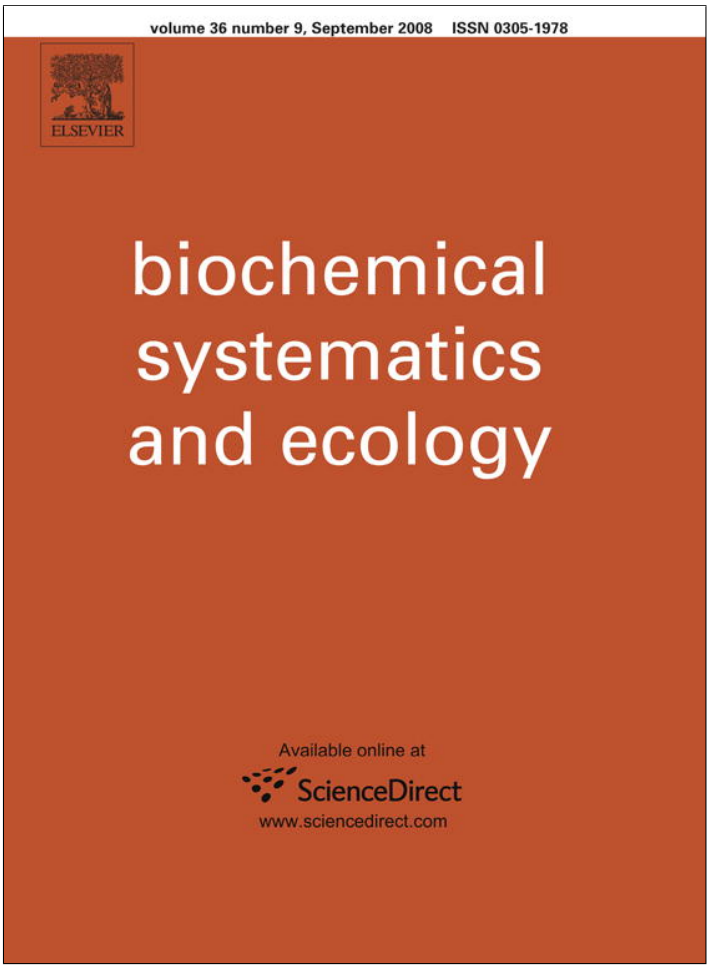

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The chemotaxonomic and medicinal significance of phenolic acids in *Arctopus* and *Alepidea* (Apiaceae subfamily Saniculoideae)

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ABSTRACT

The occurrence of (*R*)-3′-*O*-β-D-glucopyranosylrosmarinic acid, rosmarinic acid and caffeic acid in two important South African medicinal plants is reported for the first time. (*R*)-3′-*O*-β-D-Glucopyranosylrosmarinic acid and rosmarinic acid were isolated and identified in several samples from three species of the genus *Arctopus* L. (*sieketroos*) and three species of the genus *Alepidea* F. Delaroche (*ikhathazo*), both recently shown to be members of the subfamily Saniculoideae of the family Apiaceae. The compounds occur in high concentrations (up to 15.3 mg of (*R*)-3′-*O*-β-D-glucopyranosylrosmarinic acid per g dry wt) in roots of *Arctopus*. Our results provide a rationale for the traditional uses of these plants, as the identified compounds are all known for their antioxidant activity, with rosmarinic acid further contributing to a wide range of biological activities. Furthermore, we confirm the idea that (*R*)-3′-*O*-β-D-glucopyranosylrosmarinic acid is a useful chemotaxonomic marker for the subfamily Saniculoideae.

1. Introduction

The discovery of (*R*)-3′-*O*-β-D-glucopyranosylrosmarinic acid by Le Claire et al. (2005), together with other phenolic acids such as rosmarinic acid and chlorogenic acid in many *Eryngium* species, as well as in *Sanicula europaea* L. (all from the Apiaceae subfamily Saniculoideae), led to the proposal that the glycoside may be a chemotaxonomic marker for the Saniculoideae. While rosmarinic acid is commonly found in a wide range of species from different families, its glycosylated derivative, (*R*)-3′-*O*-β-D-glucopyranosylrosmarinic acid, seems to be unique to the Saniculoideae. It is not known from any other higher plants but has been isolated from cell cultures of a hornwort, *Anthoceros agrestis* Paton (Vogelsang et al., 2006).

Rosmarinic acid (**2**) occurs in numerous medicinal plants, especially of the Boraginaceae, Lamiaceae and Apiaceae, and is known for its antioxidant, antiphlogistic, astringent, anti-inflammatory, antimutagenic, antibacterial and antiviral activities (Burger and Wachter, 1998; Parnham and Kesselring, 1985; Petersen and Simmonds, 2003; Tewtrakul et al., 2003).

Based on molecular evidence, the southern African genera *Alepidea* and *Arctopus* were recently shown to be basally divergent within a broadened concept of Saniculoideae (Calviño and Downie, 2007). It was shown that *Alepidea* and *Arctopus* are the first two (earliest) lineages within the phylogeny of the subfamily. Species of both genera are very important in traditional medicine (see later). Roots of *Alepidea* and *Arctopus* contain kaurene-type diterpenoids (Holzapfel et al., 1995), known for their antimicrobial, anti-parasitic and anti-inflammatory activity (Ghisalberti, 1997). No other compounds have hitherto been identified from these genera.

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During routine chromatographic analyses of various medicinal plants used as traditional tonics, extracts of *Arctopus* and *Alepidea* showed similar and highly characteristic compounds in HPLC chromatograms. The purpose of this paper is to report on the presence of phenolic acids in samples of the two genera. We also present supporting evidence for the chemotaxonomic value of (*R*)-3′-*O*-β-D-glucopyranosylrosmarinic acid (**1**) in the subfamily Saniculoideae.

2. Materials and methods

2.1. General procedures

TLC analysis of the phenolic acids was performed with silica gel 60 F_{254} plates (Merck) and detected with 5% ethanolic sulfuric acid and 1% ethanolic vanillin (heated to 100 °C). A Shimadzu 10A HPLC instrument with a Phenomenex RP C18 column (150 × 4.6 mm, 5 μm), a binary gradient system and a photodiode array (PDA) detector was used for routine analyses. UV spectra were recorded between 210 and 400 nm. Solid phase extraction was used for sample preparation (Chromabond sorbens (ec f), Machery Nagel). C_{18} reversed-phase column chromatography was performed on a Machery Nagel Chromabond ec f column (2.5 cm × 21.0 cm). Kieselgel GF_{254} (15 μm, Merck) was used for flash chromatography. ^{1}H-NMR and ^{13}C-NMR experiments were performed on a Varian Unity Inova spectrometer operating at 300 MHz (^{1}H) and 75 MHz (^{13}C) in DMSO-d_6 for **1** and in CD_3OD for **2**, with the solvents used as internal standards in both cases. The UPLC-MS instrumentation consisted of a Waters Acquity Ultra Performance LC system equipped with PDA, a Waters LCT Premier mass spectrometer and an Acquity UPLC BEH C_{18} column (17 μm, 2.1 × 100 mm). ESI in negative ionization mode was used to determine the accurate masses of the isolated phenolic acids (**1** and **2**), and to confirm the identity of compound **3**.

2.2. Plant material

Voucher specimens were deposited in the herbarium of the Department of Botany and Plant Biotechnology at the University of Johannesburg (JRAU): AdC, A. de Castro; ARM, A.R. Magee; BEVW, B.-E. van Wyk; DKO, D.K. Olivier; JSB, J.S. Boatwright; PW, P. Winter. The samples studied are listed in Table 1 together with provenances: *Arctopus echinatus*: ARE1, PW & BEVW 170; ARE2, ARM & JSB 15; ARE3, BEVW s.n.; ARE4, BEVW 4128(a–c); ARE5, ARM & JSB 4; ARE6, DKO 66(a,b). *Arctopus dregei*: ARD7, ARM & JSB 31; ARD8, ARM & JSB 2(a–c). *Arctopus monacanthus*: ARM9, BEVW 3522; ARM10, BEVW 4161(a); ARM11, BEVW 4141(a). *Alepidea amatymbica*: ALA12, PW 252. *Alepidea comosa*: ALC13, AdC 357. *Alepidea longifolia*: ALL14, AdC 357(a,b).

2.3. Phenolic acid variation study

Air-dried samples were extracted with boiling water (0.3 g in 3 mL), treated with ethanol to remove alcohol precipitable solids and centrifuged. The dry supernatant was re-suspended in 1 mL of 50% MeOH/H_2O and loaded onto a SPE C18RP cartridge. The rosmarinic acid and its derivatives were selectively extracted with 2 mL of 30% MeOH/H_2O.

The presence of the phenolic acids was confirmed by TLC in (a) *n*-BuOH/H_2O/AcOH (4:1:2) and (b) EtOAc/MeOH/H_2O/AcOH (60:15:15:1) (Le Claire et al., 2005). R_f-values of 0.23 and 0.50 for **1** and **2** (system b) corresponded to those of Le Claire et al. (2005). The approximate concentrations of the acids were determined by HPLC analyses (flow rate 1 mL/min, linear gradient of 40–100% MeOH/1% AcOH over 30 min). Three well-resolved peaks were observed at 5.67 min, 8.23 min and 10.13 min for **3**, **1** and **2,** respectively (see Table 1).

2.4. Identification of compounds *1*, *2* and *3*

Compounds **1** (5.2 mg) and **2** (13.3 mg) were isolated from root material of *A. monacanthus* (ARM9, 24.6 g), using phase separation (30% MeOH/H_2O, absorbed on a C_{18} reversed-phase column, eluted with a MeOH/H_2O gradient (50–100%)), followed by repeated flash column chromatography (*n*-BuOH/MeOH/H_2O (4:1:1)). Compound **1** is unstable (Le Claire et al., 2005) and required further purification.

The structures of **1** and **2** were determined by utilizing 1D ^{1}H-NMR and ^{13}C-NMR experiments and COSY, HMBC and HSQC experiments. The spectroscopic data are summarized in Table 2.

The identity of compound **3** was determined by means of comparison of the mass and UV spectra, as well as the retention time of the compound with that of an authentic sample of caffeic acid (**3**). HPLC with both PDA and MS detection were used for this purpose. A gradient solvent system was used for LC/MS consisting of (A) acetonitrile and (B) 0.1% aqueous formic acid. Gradient: start, A = 5%, 3 min; A = 50%, 10 min; A = 95%, 10 min; A = 5%, 10 min; hold for 2 min.

2.4.1. (R)-3′-O-β-D-Glucopyranosylrosmarinic acid (*1*)

LC-online UV: λ_{max} = 329 nm, 251 nm. HR-TOF-ESIMS, m/z = 521.1284 $[M-H]^-$, Calculated for $C_{24}H_{25}O_{13}$: 521.1295. ^{1}H- and ^{13}C-NMR (^{1}H 300 MHz; ^{13}C 75 MHz, DMSO-d_6): see Table 2.

Table 1
Presence and approximate levels (single measurements) of (*R*)-3′-*O*-β-D-glucopyranosylrosmarinic acid (**1**), rosmarinic acid (**2**) and caffeic acid (**3**) in different species of *Arctopus* and *Alepidea* as determined by HPLC

Sample no.	Locality	**1** (mg/g)	**2** (mg/g)	**3** (mg/g)
Arctopus echinatus				
ARE1[a]	Du Toit's Kloof Pass	2.6	15.5	0.6
ARE2[a]	Hermanus	0.7	1.3	0.8
ARE3[a]	Pakhuis Pass	8.4	10.1	0.3
ARE4(a)[a]	Nieuwoudtville	0.2	2.8	1.1
ARE4(b)[a]	Nieuwoudtville	1.7	3.2	0.4
ARE4(c)[a]	Nieuwoudtville	0.2	0.8	0.4
ARE5[a]	Rondeberg	2.5	4.0	0.4
ARE6(a)[b]	Port Alfred	0.2	5.6	0.2
ARE6(a)[a]	Port Alfred	2.4	1.1	0.3
ARE6(b)[b]	Port Alfred	n.d.[c]	7.9	0.5
ARE6(b)[a]	Port Alfred	15.3	3.3	0.5
Arctopus dregei				
ARD7[a]	Malmesbury	0.5	0.3	0.4
ARD8(a)[a]	Rondeberg	4.0	11.3	1.8
ARD8(b)[a]	Rondeberg	3.1	5.0	1.5
ARD8(c)[a]	Rondeberg	6.5	14.4	1.2
Arctopus monacanthus				
ARM9[a]	Gifberg	3.9	13.1	0.4
ARM10[a]	Gifberg	3.2	1.9	3.6
ARM11[a]	Citrusdal	2.4	2.9	1.3
Alepidea amatymbica				
ALA12[b]	Mphendle	n.d.	7.3	0.4
ALA12[a]	Mphendle	0.5	9.6	0.4
Alepidea comosa				
ALC13[a]	Drummond	0.9	6.5	0.3
Alepidea longifolia				
ALL14[b]	God's Window	0.6	9.5	1.0
ALL14[a]	God's Window	0.5	14.0	0.9

[a] Roots used for extraction.
[b] Leaves used for extraction.
[c] Not detected at a detection limit of 0.1 mg/g.

2.4.2. Rosmarinic acid (**2**)

LC-online UV: $\lambda_{max} = 330$ nm, 248 nm. Negative HR-TOF-ESIMS, $m/z = 359.0772$ $[M - H]^-$. Calculated for $C_{18}H_{15}O_8$: 359.0767. ^{1}H- and ^{13}C-NMR (^{1}H 300 MHz; ^{13}C 75 MHz, CD_3OD): see Table 2.

2.4.3. Caffeic acid (**3**)

LC-online UV: $\lambda_{max} = 323$ nm, 245 nm. Negative ESIMS: $m/z = 179$ $[M - H]^-$.

3. Results and discussion

3.1. Isolation and identification of main phenolic compounds

The presence of rosmarinic acid (**2**), its glucoside (**1**), as well as caffeic acid (**3**) in *Arctopus* and *Alepidea* is reported here for the first time. The initial TLC screening of aqueous extracts of the powdered dried rhizomes of three *Arctopus* species collected in different localities revealed three pink zones, indicating the presence of phenolic compounds. Rosmarinic acid (**2**) and its glucosylated derivative (**1**) were successfully isolated. Their identities were determined as (*R*)-3′-*O*-β-D-glucopyranosylrosmarinic acid (**1**) and its aglycone, rosmarinic acid (**2**) (see Fig. 1) by means of 1D and 2D NMR which compared favorably with the NMR data from the literature (Le Claire et al., 2005; Woo and Piao, 2004). Long-range ^{1}H,^{13}C correlations observed in a HMBC experiment confirmed the identity of **1** as (*R*)-3′-*O*-β-D-glucopyranosylrosmarinic acid and not the (*R*)-3-*O*-β-D-glucopyranosylrosmarinic acid isomer by clear correlation between the anomeric proton of the glucopyranosyl unit and the oxygen bearing C-3′ on the dihydroxyphenyllactic acid ring. LC/MS and HPLC-PDA analyses proved that compound **3**, contrary to expectation (Le Claire et al., 2005), was caffeic acid ($m/z = 179$ $[M - H]^-$; UV $\lambda_{max} = 323$ nm, 245 nm) and not chlorogenic acid.

The yields of the three compounds in the different species vary greatly within populations and between localities. Compound **2** is the major constituent in most of the aqueous extracts, followed by **1**, and **3** being the minor constituent in

Table 2
^{1}H and ^{13}C NMR spectroscopic data for (*R*)-3′-*O*-β-D-glucopyranosylrosmarinic acid (**1**) and rosmarinic acid (**2**)

Position	*R*-(+)-3′-*O*-β-D-Glucopyranosyl rosmarinic acid (**1**)		Rosmarinic acid (**2**)	
	δ_H (DMSO-d_6)	δ_C (DMSO-d_6)	δ_H (MeOH-d_4)	δ_C (MeOH-d_4)
Rosmarinic acid moiety				
1		125.6		125.5
2	7.05 (d, *J* 1.5 Hz)	114.9	7.05 (d, *J* 1.8 Hz)	114.9
3		148.9		145.8
4		146.3		148.8
5	6.75 (d, *J* 9.0 Hz)	116.0	6.76 (d, *J* 8.1 Hz)	115.9
6	6.99 (dd, *J* 9.0 and 1.5 Hz)	122.1	6.99 (dd, *J* 8.4 and 2.1, Hz)	121.8
7	7.45 (d, *J* 15.0 Hz)	146.5	7.44 (d, *J* 15.9 Hz)	148.8
8	6.25 (d, *J* 15.0 Hz)	113.4	6.23 (d, *J* 15.6 Hz)	113.5
9		166.2		166.1
1′		131.4		127.7
2′	6.74 (brs)	116.5	6.67 (d, *J* 2.1 Hz)	116.8
3′		145.8		145.1
4′		144.4		144.1
5′	7.01 (d, *J* 9.0 Hz)	117.2	6.63 (d, *J* 8.1 Hz)	115.2
6′	6.65 (dd, *J* 7.5 and 1.5 Hz)	120.5	6.52 (dd, *J* 8.1 and 2.1 Hz)	120.2
7′	3.04 (dd, *J* 13.5 and 3.0 Hz) 2.95 (dd, *J* 13.5 and 9.0 Hz)	36.3	2.99 (dd, *J* 14.4 and 4.2 Hz) 2.88 (dd, *J* 14.4 and 8.4 Hz)	36.3
8′	5.07 (dd, *J* 7.5 and 6.0 Hz)	72.9	5.01 (dd, *J* 8.4 and 4.2 Hz)	73.2
9′		171.1		171.3
Glucose moiety				
1″	4.63 (d, *J* 6.0 Hz)	102.3		
2″	3.28 (m)	73.5		
3″	3.28 (m)	76.0		
4″	3.13 (m)	70.0		
5″	3.28 (m)	77.3		
6″	3.75 (m)[a] 3.45 (m)[a]	60.9		

[a] Obscured by solvent peak.

most cases. A comparison of leaves and roots from the same plant of *Arctopus echinatus* (ARE6(a) and (b)) showed that the glycoside **1** is practically absent from the leaves.

3.2. Chemotaxonomic significance of phenolic acids

Le Claire et al. (2005) suggested that the rosmarinic acid glucoside (**1**) may be a chemotaxonomic marker for the subfamily Saniculoideae of the family Apiaceae as this compound was detected in several species of the genus *Eryngium* L. and in *Sanicula europaea* L., both members of the tribe Saniculeae of the subfamily Saniculoideae. Compound **1** has not been reported from any other higher plants (but curiously, it was also found in cell cultures of a hornwort; Vogelsang et al., 2006). This compound, together with compounds **2** and **3**, were present in root samples of all three *Arctopus* species and three *Alepidea* species. While rosmarinic acid (**2**) and caffeic acid (**3**) are commonly found in medicinal plants, the occurrence of **1** in both *Arctopus* and *Alepidea* agrees with molecular systematic evidence (e.g. Calviño and Downie, 2007) that the two genera are related. (*R*)-3′-*O*-β-D-Glucopyranosylrosmarinic acid is thus confirmed to be a chemotaxonomic marker for the Saniculoideae, tribe Saniculeae. It may be interesting to extend this study to other genera of this tribe for which data on the presence of **1** is currently not available, as well as for the newly described tribe Steganotaeniae (Calviño and Downie, 2007).

3.3. Medicinal significance of phenolic acids

Species of *Arctopus* and *Alepidea* are very important in southern African traditional medicine (De Castro and Van Wyk, 1994; Hutchings et al., 1996; Magee et al., 2007; Van Wyk et al., 2000). Rhizomes of *Alepidea amatymbica* Eckl. & Zeyh. (known as *ikhathazo* in Zulu) and other species are widely used to treat respiratory ailments during the winter period and are commonly sold at traditional muthi markets (De Castro and Van Wyk, 1994; Hutchings et al., 1996). Tubers of *Arctopus echinatus* and *A. monacanthus* (*sieketroost* or *platdoorn* in Dutch) are well known as important Khoi-San and Cape Dutch traditional medicines and general tonics, used for a wide range of ailments that includes venereal diseases and respiratory ailments (Magee et al., 2007). They are also used by epilepsy sufferers and have sedative properties (Stafford et al., 2005). The medicinal uses of *Arctopus* and *Alepidea* species were hitherto ascribed to the presence of kaurene-type diterpenoids (Holzapfel et al., 1995; Van Wyk et al., 2000) so that the presence of phenolic acids adds a new dimension to our understanding of the medicinal value and possible efficacy of these plants. As mentioned in Section 1, the medicinal value of

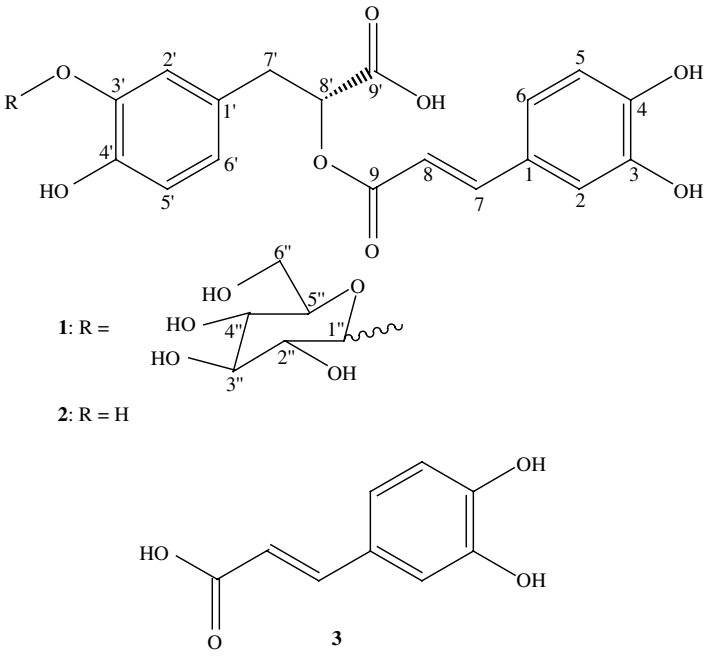

Fig. 1. Structures of phenolic acids: **1**, (*R*)-3′-*O*-β-D-glucopyranosylrosmarinic acid; **2**, rosmarinic acid; **3**, caffeic acid.

phenolic acids (and especially rosmarinic acid) are ascribed to antioxidant, antiphlogistic, astringent, anti-inflammatory, antimutagenic, antibacterial and antiviral activities (Burger and Wachter, 1998; Parnham and Kesselring, 1985; Petersen and Simmonds, 2003). The traditional uses of *Alepidea* and *Arctopus* species (especially as general tonics and for treating respiratory ailments) are therefore strongly supported by the confirmed presence of relatively high levels of phenolic acids in the rhizomes and tubers (i.e., the parts that are used medicinally). It is interesting to note that the European *Sanicula europaea*, *Eryngium campestre* L. and *Eryngium planum* L. are used in much the same way (Burger and Wachter, 1998; Van Wyk and Wink, 2004). In the case of *Sanicula europaea* (sanicle herb, *saniculae herba*), the leaves are mainly used medicinally and it is therefore noteworthy that Le Claire et al. (2005) reported high levels of rosmarinic acid and its glycoside in leaves of this species. The German Commission E allows for sanicle herb the indication "mild cattarhs of the respiratory tract" (Blumenthal et al., 1998). The medicinal members of the subfamily Saniculoideae therefore represent an interesting example of related and chemically similar plants that are used in different parts of the world for the same or similar treatments.

4. Conclusion

The presence of phenolic acids, and especially rosmarinic acid and its glucoside, are useful chemotaxonomic markers for members of the tribe Saniculeae (subfamily Saniculoideae of the Apiaceae). The close relation between the morphologically anomalous *Arctopus* and *Alepidea* and the genera *Eryngium* and *Sanicula* suggested by molecular systematic evidence is supported by the unique presence of the rosmarinic acid glycoside in these genera. Phenolic acids were not previously known from *Arctopus* and *Alepidea*, so that their presence provides new supporting evidence for the traditional medicinal uses of these African plants, especially when the similarity of uses of the European members of the same tribe and subfamily are considered.

Acknowledgements

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