Notes on the occurrence and significance of triterpenoids (asiaticoside and related compounds) and caffeoylquinic acids in *Centella* species

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Abstract

Asiaticoside, a well-known ursane-type triterpene saponin widely used as a chemical marker in quality control of commercial samples of *Centella asiatica,* occurs in South African material (12 samples from four provenances) at a level of 1 to 2% of dry weight. Surprisingly, the compound was not detected in three samples of *C. glabrata*, the only other species with a well-recorded history of medicinal use. A preliminary LC-MS survey of a further 21 leaf samples from 16 species of *Centella* revealed the presence of asiaticoside as a major constituent in ten of the species studied, while others had various isomers of chlorogenic and dicaffeoylquinic acids. Multiple samples from the same species were chemically similar, indicating that a wider chemosystematic study of the entire genus may yield valuable results. The study also showed that South African genotypes of *C. asiatica* may be suitable for commercial use and that several other *Centella* species are potential sources of asiaticoside.
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1. Introduction

Centella L. is a genus of ca. 45 species of the family Apiaceae (Umbelliferae) and belongs to the subfamily Mackinlayoideae. All but one species [*C. asiatica* (L.) Urb.] are endemic to southern Africa. An as yet unpublished revision of the genus (Schubert, 2000) showed that three distinct subgenera can be distinguished, based mainly on the sex of the florets: 1, *Trisanthus* (Lour.) Drude, with all flowers hermaphroditic (*C. asiatica* only); 2, *Solandra* (L.) Drude, with a single central hermaphroditic flower surrounded by male umbellules [four species, including *C. capensis* (L.) Domin]; 3, *Centella*, with functionally female and male florets on the same or on different plants, i.e., andromonoecious and/or androdioecious (the remaining 40 species). Relationships among the species are poorly known and the delimitation of taxa is often problematic.

C. asiatica [*Hydrocotyle asiatica* L., *gotu kola*, Indian pennywort, *brahma-manduki* and *brahmi-buti* (Hindi), *tsubo-kusa* (Japanese), *tungchian* and *luei gong gen* (Chinese)] is internationally well known as a medicinal plant. It has a pan-tropical distribution and is used in many healing cultures, including Ayurvedic medicine, Chinese traditional medicine, Kampo (Japanese traditional medicine) and African traditional medicine (Brinkhaus et al., 2000). One of the main active constituents of *C. asiatica* is the ursane-type triterpene saponin, asiaticoside, which is responsible for wound healing properties (Kim et al., 2009; Shukla et al., 1999) and is known to stimulate type 1 collagen synthesis in fibroblast cells (Lee et al., 2006). Plants collected from various geographical regions and locations in India, Madagascar, Malaysia, Sri Lanka, Andaman Islands and South Africa have yielded concentrations of asiaticoside ranging from 0.006 to 6.42% of dry weight (Aziz et al., 2007; Das and Mallick, 1991; Günter and Wagner, 1996; James et al., 2008; Rafamantanana et al., 2009; Randriamampionona et al., 2007; Schaneberg et al., 2003; Thomas et al., 2010). *C. asiatica* also contains several other triterpene saponins. Madecassoside always co-occurs with asiaticoside as a main compound and other

lesser saponins have been reported, such as asiaticosides A to G, centelloside, brahmoside and many others (Combined Chemical Dictionary, 2012; Nhiem et al., 2011; Singh et al., 2010).

Two species of *Centella* are widely used in traditional medicine in South Africa, namely *C. asiatica* and *C. glabrata* L. *C. asiatica* is used mainly for wound healing, burns, ulcers, leprosy, tuberculosis, lupus, skin diseases, eye diseases, fever, inflammation, asthma, hypertension, rheumatism, syphilis, epilepsy, diarrhea and mental illness (Jiang et al., 2005; Neuwinger, 2000; Nhiem et al., 2011; Shen et al., 2009; Van Wyk and Wink, 2004; Van Wyk et al., 2009) but is also eaten as a vegetable (Van Wyk and Gericke, 2000; Van Wyk et al., 2008) or used as a spice (Jiang et al., 2005). *C. glabrata* is an Old Cape remedy for diarrhea and dysentery (Kling, 1923; Pappe, 1847, 1850, 1857, 1868; Smith, 1966; Watt and Breyer-Brandwijk, 1962). A summary of all recorded ethnobotanical uses of *Centella* species in Africa is given in Table 1.

The aim of this study was to explore the potential value of chemical compounds as chemotaxonomic markers in the genus *Centella* and to investigate the occurrence and diagnostic value of asiaticoside and other triterpene saponins, with emphasis on the two species of medicinal interest (*C. asiatica* and *C. glabrata*).

2. Materials and methods

2.1. Materials used

Asiaticoside, asiatic acid and chlorogenic acid supplied by Sigma-Aldrich (Johannesburg, South Africa) were used as reference standards. Asiaticoside was used as an external standard for quantification of the compound in 12 *C. asiatica* samples. Acetonitrile (Romil far UV grade) was supplied by Microsep (Johannesburg, South Africa), analytically pure formic acid was acquired from Sigma-Aldrich (Johannesburg, South Africa), and ultrapure water was used.

The species studied, together with provenances and/or voucher specimen numbers are listed in Table 2. Voucher specimens have been deposited in the University of Johannesburg Herbarium (JRAU). All samples were rapidly air-dried after collection and either extracted immediately or stored under cool, dry and dark conditions for future studies. Most of the samples of *C. asiatica* (used for the quantification of asiaticoside) were collected fresh (February 2012) but some old samples were available for studying the effects of age and storage. Each population was sampled in triplicate (i.e., three different

Table 1
Recorded ethnobotanical uses of *Centella* species in Africa.

Centella asiatica (L.) Urb.	Reference
Wounds and sores are treated topically. Used as a remedy for leprosy.	Pappe (1857, 1868)
Leprosy is treated with this herb. Topical application to wounds and sores is made.	Kling (1923)
The plant is used to treat leprosy and lupus in India and Madagascar and to treat leprosy in the Cape and in central Africa. It has been used experimentally for treating tuberculosis and syphilis. The fresh herb has been used as a cooling, diuretic and purgative treatment for leprosy and syphilis in southern Asia. The fresh herb is applied to wounds and the dried herb is used as a snuff. The plant has been used for fevers, bowel complaints and for syphilitic and scrofulous conditions and was thought to have been narcotic. The leaf is eaten as a food by the Xhosa and the Mfengu.	Watt and Breyer-Brandwijk (1962)
Softened warmed leaves are applied directly to sores for six days. Leaves are also crushed by rubbing in the hands to form a pulp which is applied to the wound and bandaged into place. The leaves are used to make a tea which acts as a diuretic, light purgative, febrifuge and as a wash for sores and wounds, mouth infections and ulcers. The tea is also used to treat tuberculosis. The dried leaf is used as a snuff.	Roberts (1990)
Leaves are ground and applied to the skin. Skin complaints are often treated with this plant.	Hutchings et al. (1996)
Leaf sap is taken orally to treat amoebic dysentery and menorrhagia, applied into the ear for suppurating otitis, applied on wounds, and dropped into the eye to calm the insane. A leaf maceration is drunk for coughs and leaf decoctions are drunk as a tonic for pregnant women and for oxytocic conditions. A leaf extract is drunk for gastric ulcers and liver inflammation. Leaf pulp or leaf powder is applied to wounds. Leaf powder is used for skin ailments, leprosy, peptic ulcers, acne and allergic skin rashes. Leaf pulp is applied to syphilitic ulcers and furuncles. Leaf tea is drunk for syphilitic sores and gastric ulcers. Fresh leaves are also chewed for gastric ulcers. The boiled plant is bound to the chest as a galactagogue. A plant decoction is added to bath water as a tonic for young children with malaria.	Neuwinger (2000)
The leaves are cooked as a spinach.	Van Wyk and Gericke (2000)
Severe wounds and syphilitic ulcers are healed by applying heated or crushed leaves as poultices. A leaf tea acts as a mouthwash for ulcers and infections and has a diuretic, purgative and anti-pyretic action.	Von Koenen (2001)
Fresh leaves are used as ear plugs for ear pain in children. Children also eat the fresh leaves.	Van Wyk et al. (2008)
Leaves are cooked as a spinach, mixed with mealie-meal and salt and eaten.	Fox and Norwood Young (1982)
Centella glabrata L.	
A decoction of the roots and stalks is used to treat violent diarrhea and chronic dysentery. The common name is "persgras".	Pappe (1847, 1850, 1857, 1868)
The common name for *C. glabrata* is "persgras".	Marloth (1917)
A tea or an extract of the plant is used to treat chronic diarrhea and dysentery. The plant is known as "persgras".	Kling (1923)
A decoction of the root, stem and leaves was used for diarrhea and chronic dysentery by the early Cape farmers.	Watt and Breyer-Brandwijk (1962)
The plant is said to induce perspiration. The name "persiegras" is derived from the old Cape Dutch term "persi" meaning dysentery. Decoctions of the leaves and stalks were used for diarrhea and dysentery.	Smith (1966)
Whole plants are used as traditional medicine (unspecified).	Cunningham (1988), Hutchings et al. (1996)

Table 2
Species and provenance/voucher specimen details of 33 samples from 16 species of *Centella* screened for the presence of asiaticoside and madecassoside. ND = not determined (+ = major compound; – not detected or trace amounts); SD based on three injections per sample.

Samples and species	Provenance and/or voucher specimen (all in JRAU)	Date collected	Asiaticoside presence as major compound (% dry wt. determined for samples 1 to 12 only)		Standard deviation	Madecassoside (presence as major compound only, not quantified)
1. *C. asiatica*	Port Edward, plant 1	Mar 2008	+	1.37	0.02	+
2. *C. asiatica*	Port Edward, plant 2	Mar 2008	+	1.64	0.03	+
3. *C. asiatica*	Port Edward, plant 3	Mar 2008	+	1.48	0.03	+
4. *C. asiatica*	Still Bay, plant 1	Feb 2012	+	1.22	0.11	+
5. *C. asiatica*	Still Bay, plant 2	Feb 2012	+	0.98	0.05	+
6. *C. asiatica*	Still Bay, plant 3	Feb 2012	+	1.24	0.09	+
7. *C. asiatica*	Swellendam, plant 1	Feb 2012	+	1.89	0.03	+
8. *C. asiatica*	Swellendam, plant 2	Feb 2012	+	2.06	0.06	+
9. *C. asiatica*	Swellendam, plant 3	Feb 2012	+	1.40	0.05	+
10. *C. asiatica*	Table Mountain, plant 1	Feb 2012	+	1.78	0.02	+
11. *C. asiatica*	Table Mountain, plant 2	Feb 2012	+	1.72	0.11	+
12. *C. asiatica*	Table Mountain, plant 3	Feb 2012	+	0.75	0.04	+
13. *C. asiatica*	*Ex. hort.*, Van Wyk, *Long 1*	Oct 2007	+	ND		+
14. *C. asiatica*	Klipriviersberg, *Long 13*	Jan 2008	+	ND		+
15. *C. asiatica*	Loskop Dam, *De Castro 279*	Jan 1993	+	ND		+
16. *C. calliodus*	Harold Porter, *Van Wyk 3567*	Apr 1994	+	ND		+
17. *C. calliodus*	Bain's Kloof, *Schubert & Van Wyk 32*	Sep 1994	+	ND		+
18. *C. capensis*	Lion's Head, *Schubert & Van Wyk 47*	Sep 1994	+	ND		+
19. *C. difformis*	Harold Porter, *Schubert & Van Wyk 23*	Sep 1994	+	ND		+
20. *C. eriantha*	Ruiterbos, *Van Wyk 3561*	Apr 1994	–	ND		–
21. *C. flexuosa*	Franschhoek Pass, *Schubert & Van Wyk 28*	Sep 1994	+	ND		+
22. *C. fusca*	Elandskloof, *Schubert & Van Wyk 62*	Sep 1994	+	ND		+
23. *C. glabrata*	Paarl Mountain Road, *Van Wyk 4363*	May 2011	–	ND		–
24. *C. glabrata*	Franschhoek Pass, *Van Wyk 4364*	Jun 2009	–	ND		–
25. *C. glabrata*	Du Toit's Kloof, *Schubert 11*	Sep 1994	–	ND		–
26. *C. glauca*	Dasklip Pass, *Schubert & Van Wyk 101*	Apr 1995	+	ND		+
27. *C. lanata*	Montagu Pass, *Long 8*	Sep 2009	–	ND		–
28. *C. scabra*	Elandskloof Pass, *Schubert & Van Wyk 66*	Sep 1994	+	ND		+
29. *C. thesioides*	Jonaskop, *Schubert & Van Wyk 34*	Sep 1994	–	ND		–
30. *C. tridentata*	Palmiet River, *Schubert & Van Wyk 19*	Sep 1994	+	ND		+
31. *C. triloba*	Kogel Bay, *Schubert & Van Wyk 15*	Sep 1994	+	ND		+
32. *C. villosa*	Franschhoek Pass, *Schubert & Van Wyk 30*	Sep 1994	–	ND		–
33. *C. virgata*	Tradouw Pass, *Schubert & Van Wyk 67*	Sep 1994	–	ND		–

individual plants were sampled). Three populations of *C. glabrata* and two populations of *C. calliodus* were sampled to study possible geographical variation. Several other samples from a previous taxonomic study (Schubert, 2000) were available for further comparative studies. These samples were mostly collected in the same month and carefully stored under cool, dry conditions.

2.2. Sample preparation

Air-dried plant material (leaves and aboveground stems) was powdered and extracted for 15 min at 60 °C in methanol at a ratio of 1 g/10 ml. After filtration the samples were dried by evaporation. This extraction method was compared with a second method, which was finally chosen for the accurate determination of the asiaticoside content. Air-dried plant material was powdered and extracted with 50% acetonitrile, 49% water and 1% formic acid at a ratio of 1 g/20 ml. The samples were vortexed, left overnight at room temperature, placed in an ultrasonic bath for 1 h, filtered and dried.

Samples were reconstituted in 50% acetonitrile, 49% water and 1% formic acid in varying volumes to yield a final concentration of exactly 500 mg original dry leaf weight in 10 ml solvent. The samples were diluted a further 10 times and a 3 µl injection volume was used.

2.3. Liquid chromatography mass spectrometry (LC–MS) analysis

LC–MS analyses were performed on a Waters (Milford, MA, USA) Synapt G2 quadrupole time of flight mass spectrometer coupled to a Waters Acquity ultraperformance liquid chromatograph (UPLC) fitted with an Acquity photo diode array (PDA) detector. Separation was achieved on a Waters UPLC BEH C18

column (2.1 × 100 mm, 1.7 μm particle size) with 0.1% formic acid as mobile phase A and acetonitrile as mobile phase B. The flow rate was 0.35 ml per min. The gradient that was employed started for the first 30 s at 100% solvent A followed by a concave gradient (curve 7 setting on Waters Masslynx software) to 100% B over the next 16.5 min. The column was washed for a min in 100% B, followed by re-equilibration to the starting conditions for 2 min.

Electrospray ionization was applied in the negative mode at a capillary voltage of 2.5 kV, cone voltage of 25 V, desolvation temperature of 275 °C and desolvation gas setting of 650 L/h. The rest of the MS settings were optimized for best sensitivity. The instrument was calibrated with sodium formate and leucine enkaphelin was used as lock mass for accurate mass determinations. The MS acquisition method consisted of a low energy function at a trap voltage of 6 V and a high energy function where the trap collision energy was ramped from 15 to 60 V to generate fragmentation data (MS^E).

2.4. Data processing

Waters Masslynx version 4.1 software was used to process the data. The Targetlynx application manager of Masslynx was used to quantify the asiaticoside, based on the calibration equation y = 183.63 × (R^2 = 0.998, using a five point calibration curve, with the following concentrations of the asiaticoside standard: 0.02 to 60 ppm). The LOQ value was 20 ppb. The concentration of asiaticoside in the samples was high and well above the detection limit of the method (10 ppb, signal to noise of 10), so that the samples had to be diluted ten times to fall in the linear range of the MS detector (10 ppb to 100 ppm). Samples were injected three times.

3. Results

3.1. Asiaticoside content

The presence and levels of asiaticoside in 33 samples from 16 species of Centella are listed in Table 2. The concentration of asiaticoside was determined in 12 of the samples of C. asiatica and varied between 0.75 and 2.06% dry weight. Asiaticoside and madecassoside were present as major compounds in all the samples of C. asiatica and nine other species (here reported for the first time), including C. calliodus (Cham. & Schlechtd.) Drude, C. capensis, C. difformis (Eckl. & Zeyh.) Adamson, C. flexuosa, Drude, C. fusca (Eckl. & Zeyh.) Adamson, C. glauca M.T.R.Schub. & B.-E.van Wyk, C. scabra Adamson, C. tridentata (L.f.) Domin and C. triloba (Thunb.) Drude (Table 2).

3.2. Diagnostic value of main compounds

C. asiatica and C. glabrata differ markedly in the relative quantities of the major constituents, with asiaticoside and madecassoside as main compounds only in C. asiatica (Fig. 1). The presence of asiatic acid in C. asiatica and chlorogenic acid in both species (Fig. 1) was confirmed by authentic reference standards. Chlorogenic acid occurs in very high levels in C. glabrata and a base peak of m/z 707 was observed due to the formation of dimers during the electrospray ionization. A dimer is also present in the spectrum standard and the C. asiatica sample, but not as the base peak due to its lower concentration.

C. glabrata has an as yet unidentified triterpenoid as main compound, together with eight phenolic acids (Fig. 1), seven of which were also detected in C. asiatica. Irbic acid appears to be confined to C. asiatica (Fig. 1). The identity of the chlorogenic
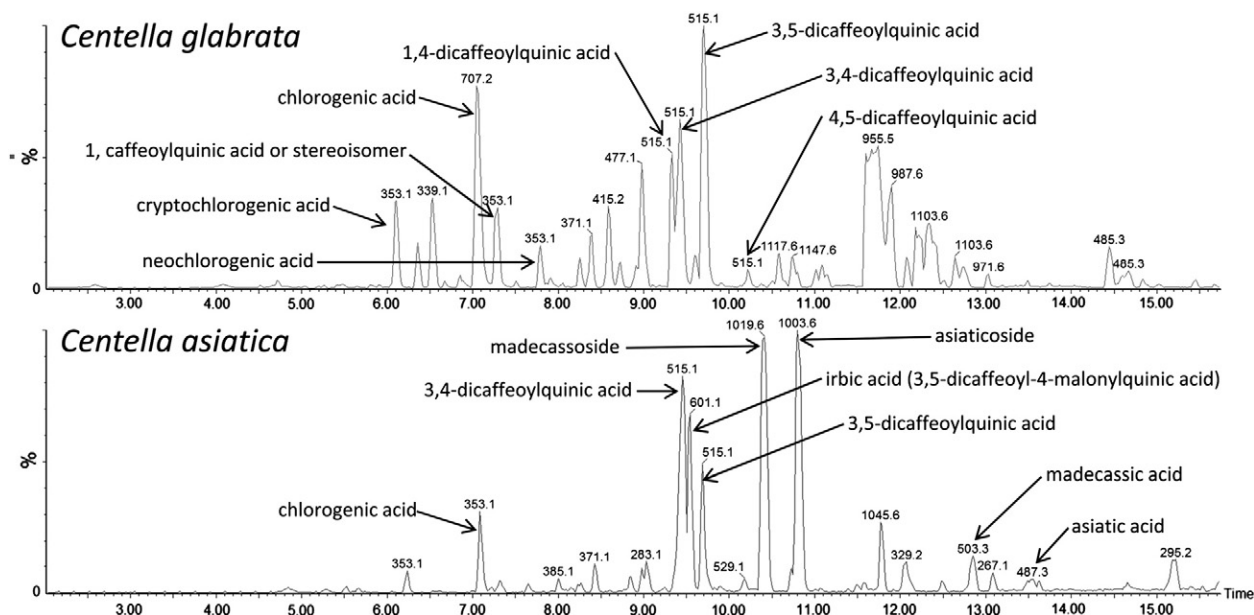

Fig. 1. LC–MS chromatograms of extracts from leaves and stems of Centella glabrata (Van Wyk 4363) and C. asiatica (ex hort., Van Wyk, Long 1).

acid isomers could be established by comparisons with the LC–MS/MS data presented by Fang et al. (2002) as well as comparisons of their relative elution order. The isomers of dicaffeoylquinic acid were tentatively identified based on their relative retention times and their different fragmentation behavior using LC–MS. The ease of the removal of the caffeoyl residue during fragmentation was proven by Clifford et al. (2005) to be 1~5>3>4.

Asiaticoside, asiatic acid and chlorogenic acid were the only compounds of those tentatively identified that were confirmed with authenticated standards. The rest of the compounds were identified based on the predicted elemental composition obtained from the accurate mass data (Table 3). The published data on *Centella* was then compared to these formulas (Clifford et al., 2005; Fang et al., 2002). The results also correspond with their UV spectra obtained from the PDA detector. With the exception of five of the phenolic acids, all compounds identified were previously reported from *C. asiatica*.

The LC–MS spectra of multiple samples of the same species (*C. asiatica*, *C. glabrata* and *C. calliodus*) were closely similar, despite large differences in geographical locality, date of collection and duration of storage.

4. Discussion

A large number of chemical compounds are known from *C. asiatica* (Brinkhaus et al., 2000; Günter and Wagner, 1996; Singh et al., 2010). These include ursane-type triterpenoids (mainly asiaticoside and madecassoside) and their aglycones (respectively asiatic acid and madecassic acid), caffeic acid derivatives [mentioned by Günter and Wagner (1996) and some compounds tentatively identified in this study], polyacetylenes such as cadiyenol (Govindan et al., 2007; Siddiqui et al., 2007), flavonoids, including quercetin and kaempferol derivatives (Razmovski-Naumovski et al., 2010; Subban et al., 2008) and mono- and sesquiterpenes in the essential oil, such as β-caryophyllene, trans-β-farnesene, terpene acetate and germacrene-D (Asakawa et al., 1982; Brinkhaus et al., 2000; Günter and Wagner, 1996). In a study of essential oil from South African material, Oyedeji and Afolayan (2005) reported that α-humulene, β-caryophyllene, bicyclogermacrene, germacrene-B and myrcene are the major constituents.

In all the South African *C. asiatica* samples, the main triterpenoid saponins were asiaticoside and madecassoside, with much smaller amounts of their respective aglycones, asiatic acid and madecassic acid. Asiaticoside invariably co-occurs with madecassoside and these two structurally related saponins are present at more or less the same concentrations. Asiaticoside is widely used as a convenient marker compound for quality control purposes (Bonfill et al., 2006; Kite et al., 2007; Liu et al., 2010; Sahu et al., 1989; Shen et al., 2009; Zhang et al., 2008). The levels of asiaticoside in the four populations studied (about 1–2% dry weight) are comparable to data for samples from other regions of the world. Indian material yielded 0.006–0.114%

Table 3

Compounds detected in *Centella glabrata* and *C. asiatica* with their accurate mass, predicted formula, major MS^E fragments and tentative identification (see Fig. 1). References: A = Antognoni et al. (2011); C = Clifford et al. (2005); F = Fang et al. (2002); R = Randriamampionona et al. (2007).

Retention time, observed accurate mass	Predicted formula	MS^E fragments	Tentatively identified as:	Confirmed with reference sample	Present in *C. asiatica*	Present in *C. glabrata*	Reference
6.1 min, m/z 353.0873	$C_{16}H_{17}O_9$ $(M-H)^-$	191, 179, 135	Cryptochlorogenic acid	−	+	+	F
6.99 min, m/z 353.0879	$C_{16}H_{17}O_9$ $(M-H)^-$	191	Chlorogenic acid	+	+	+	A, F
7.3 min, m/z 353.0867	$C_{16}H_{17}O_9$ $(M-H)^-$	191, 179, 173, 135	1-Caffeoylquinic acid or stereoisomer of chlorogenic acid	−	+	+	F
7.8 min, m/z 353.0873	$C_{16}H_{17}O_9$ $(M-H)^-$	191, 179	Neochlorogenic acid	−	+	+	F
9.35 min, m/z 515.1207	$C_{25}H_{23}O_{12}$ $(M-H)^-$	353, 191, 179, 173, 161, 135	1,4-Dicaffeoylquinic acid	−	−	+	F
9.4 min, m/z 515.1200	$C_{25}H_{23}O_{12}$ $(M-H)^-$	353, 191, 179, 135	3,4-Dicaffeoylquinic acid	−	+	+	A, C
9.6 min, m/z 601.1193	$C_{28}H_{25}O_{15}$ $(M-H)^-$	395, 233, 191, 179	3,5-Dicaffeoyl-4-malonylquinic acid (Irbic acid)	−	+	−	A
9.7 min, m/z 515.1193	$C_{25}H_{23}O_{12}$ $(M-H)^-$	353, 191, 179, 173, 135	3,5-Dicaffeoylquinic acid (Isochlorogenic acid a)	−	+	+	A, C
10.4 min, m/z 1019.5071	$C_{49}H_{79}O_{22}$ (M+formate)$^-$	974, 503, 469, 367	Madecassoside	−	+	−	R
10.8 min, m/z 1003.5108	$C_{49}H_{79}O_{21}$ (M+formate)$^-$	957, 487, 469, 367	Asiaticoside	+	+	−	R
12.8 min, m/z 503.3363	$C_{30}H_{47}O_6$ $(M-H)^-$	503.3 (major), 248.9, 118	Madecassic acid	−	+	−	R
13.5 min, m/z 487.3437	$C_{30}H_{47}O_5$ $(M-H)^-$	487.3 (major), 311.2, 265.1, 183.0	Asiatic acid	+	+	−	R

dry weight (Das and Mallick, 1991); Madagascar material, reported to be the highest in the world, 2.67–6.42% dry weight (Randriamampionona et al., 2007) or 1.63–2.0% using a validated method (Rafamantanana et al., 2009); Sri Lanka 0.15% (Schaneberg et al., 2003); Malaysia 0.79–1.15 (Aziz et al., 2007); South India and the Andaman Islands 0.02–1.70% (Thomas et al., 2010) and South Africa (Gauteng Province) 4.52 and 5.23% dry weight (James et al., 2008). Commercial samples (without specified provenances) yielded 0.18–0.52% (Günter and Wagner, 1996) or 0.76–1.01 (Schaneberg et al., 2003). At least some of the variation observed in our study (and that reported in the literature) may be ascribed to varying proportions of leaf and stem material extracted. Günter and Wagner (1996) reported that leaf material had substantially higher levels of madecassoside than stalks and woody parts. Rafamantanana et al. (2009) cautioned that the high levels reported in some studies were not obtained by validated methods. Our results agree almost perfectly with their values of 1.63–2.0% dry weight. It therefore seems that South African material of the species is suitable for commercial use.

The only compound thus far reported from *C. glabrata* is a polyacetylene (polyine), 8-acetoxyfalcarinol (Bohlmann and Zdero, 1975), which occurred as a major constituent in five species of *Centella* investigated, including *C. difformis*, *C. eriantha* Drude, *C. glabrata*, *C. sessilis* Adamson and *C. virgata* (L.f.) Drude. Our LC–MS methods were optimized for polar compounds and were therefore not suitable for the separation and detection of polyacetylenes.

C. asiatica and *C. glabrata* appear to be chemically very different, with an almost complete absence of asiaticoside and madecassoside in the latter, apparently replaced by an as yet unidentified triterpenoid with *m/z* 955 (Fig. 1). Both species, however, accumulate phenolic acids, mainly caffeoyl derivatives such as 3,5-dicaffeoylquinic acid (isochlorogenic acid A), 3,4-dicaffeoylquinic acid and chlorogenic acid (Fig. 1). Multiple samples of these two species were remarkably similar in chemical composition, regardless of provenance, time and date of collection or duration of storage. Despite obvious differences between species, the overall chemical pattern in the 16 species studied supports a wide generic concept. Some uncertainty has remained about the taxonomic status of the three subgenera proposed by Schubert (2000). The major morphological discontinuities between the subgenera, especially in their reproductive structures are not reflected in the chemical data because representatives of all three subgenera have asiaticoside and madecassoside as main compounds. A similar overlap between subgenera occurs in the presence of caffeoyl derivatives, providing further support for the idea of a relationship between seemingly unrelated species (note the similarity between *C. asiatica* and *C. glabrata*, for example — Fig. 1). The difference between species are mostly quantitative only, but there are some qualitative differences (Fig. 1; Table 3), including the apparent absence of one (1,4-dicaffeoylquinic acid) of the eight organic acids detected in *C. glabrata* that was not found in *C. asiatica*. Also noteworthy is the presence of irbic acid (3,5-dicaffeoyl-4-malonylquinic acid) in *C. asiatica* (but not in *C. glabrata*), previously known only from callus tissue and cell suspensions of this species (Antognoni et al., 2011).

Perhaps the most interesting result is the marked differences between *Centella* species which indicate that a detailed chemosystematic study using LC–MS may help to solve some of the problematic species delimitations and thus contribute to a better understanding of relationships in a taxonomically difficult genus.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.sajb.2012.07.017.

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