


Centella asiatica: phytochemistry and mechanisms of neuroprotection and cognitive enhancement

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Abstract This review describes in detail the phytochemistry and neurological effects of the medicinal herb *Centella asiatica* (L.) Urban. *C. asiatica* is a small perennial plant that grows in moist, tropical and sub-tropical regions throughout the world. Phytochemicals identified from *C. asiatica* to date include isoprenoids (sesquiterpenes, plant sterols, pentacyclic triterpenoids and saponins) and phenylpropanoid derivatives (eugenol derivatives, caffeoylquinic acids, and flavonoids). Contemporary methods for fingerprinting and characterization of compounds in *C.*

asiatica extracts include liquid chromatography and/or ion mobility spectrometry in conjunction with high-resolution mass spectrometry. Multiple studies in rodent models, and a limited number of human studies, support *C. asiatica*'s traditional reputation as a cognitive enhancer, as well as its anxiolytic and anticonvulsant effects. Neuroprotective effects of *C. asiatica* are seen in several in vitro models, for example against beta amyloid toxicity, and appear to be associated with increased mitochondrial activity, improved antioxidant status, and/or inhibition of the pro-inflammatory enzyme, phospholipase A2. Neurotropic effects of *C. asiatica* include increased dendritic arborization and synaptogenesis, and may be due to modulations of signal transduction pathways such as ERK1/2 and Akt. Many of these neurotropic and neuroprotective properties of *C. asiatica* have been associated with the triterpene compounds asiatic acid, asiaticoside and madecassoside. More recently, caffeoylquinic acids are emerging as a second important group of active compounds in *C. asiatica*, with the potential of enhancing the Nrf2-antioxidant response pathway. The absorption, distribution, metabolism and excretion of the triterpenes, caffeoylquinic acids and flavonoids found in *C. asiatica* have been studied in humans and animal models, and the compounds or their metabolites found in the brain. This review highlights the remarkable potential for *C. asiatica* extracts and derivatives to be used in the treatment of neurological conditions, and considers the further research needed to actualize this possibility.

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Introduction

Taxonomy, nomenclature and synonyms

Centella asiatica (L.) Urban is a member of the plant family Apiaceae (formerly Umbelliferae), and subfamily Mackinlayoideae (USDA 2016). The plant was reassigned from the subfamily Hydrocotyloideae as a result of molecular phylogeny studies (Nicolas and Plunkett 2009). Synonyms for *C. asiatica* are *Hydrocotyle asiatica* L. and *Hydrocotyle erecta* L.f. (USDA 2016). The name *H. asiatica* is often used in older materia medica, herbal textbooks and research publications. Almost 80 species of *Centella* have been recorded, many of which have older synonyms under the genus *Hydrocotyle* (IPNI 2016). It is therefore advisable that literature searches on *Centella* species encompass the equivalent *Hydrocotyle* nomenclature (IPNI 2016) where appropriate. Common English names for *C. asiatica* are Indian Pennywort and Asian Pennywort (IPNI 2016). In the United States and other Western countries, the herb of commerce, and dietary supplements derived from it, are commonly referred to by the Sri Lankan Sinhalese name “gotu kola” (Nadkarni 1976; Newall et al. 1996). Common names for *C. asiatica* from various parts of the world are listed in Table 1.

Botany and geographical distribution

Centella asiatica is a perennial creeping plant which propagates by producing stolons. The plants consist of shovel or spade shaped leaves with scalloped edges, borne on long petioles clustered at the stem nodes (Fig. 1). The insignificant green or pinkish-white flowers are borne in dense umbels, and the seeds are pumpkin shaped nutlets 3–5 mm in length (Floridata 2016). This plant grows in swampy areas of tropical and subtropical regions of the world (James and Dubery 2009; Long et al. 2012; USDA 2016). It is

native to Asian tropical regions of the Indian Subcontinent, South East Asia, Malaysia and the Solomon Islands, as well as some temperate regions in China, Japan, Korea and Taiwan.

C. asiatica is also native to African countries south of the equator, some regions in East, West, and West Central Africa, and endogenous to New South Wales in Australia and some Pacific Islands. The herb has been naturalized to several other regions of the world including the African islands of Madagascar and Seychelles, some temperate regions of Asia (e.g. the Caucasus), and several regions of South Eastern United States and South Central America (Mexico, Ecuador, Colombia and Venezuela). It is used as a modern or traditional botanical remedy in many of these areas and also finds culinary use as a salad vegetable, or in juices, in several Asian countries (Plengmuankhae and Tantitadapitak 2015). Regional common names are listed in Table 1.

Medicinal uses of *C. asiatica*

History and traditional uses

Brinkhaus et al. (2000) have reviewed in detail the long history of the medicinal applications of *C. asiatica*, from earliest evidence of its use by the Indian physician Sushruta (ca. 1200 BC), to traditional herbal medicine systems in Asian and African countries, its introduction to, and scientific study in Europe in the 19th and 20th centuries, and present worldwide use of the herb or its derivatives, in commercial topical and oral products (Brinkhaus et al. 2000). In several regions of the world, preparations of the herb were applied topically to treat infectious skin diseases and accelerate healing of skin ulcers and wounds, while internal preparations were used to treat severe dysentery, gastric ulcers and syphilitic lesions (Brinkhaus et al. 2000; Long et al. 2012; Nadkarni 1976). Of particular relevance to this review however, is *C. asiatica*'s importance in the Ayurvedic tradition as a “medhya-rasayana” herb i.e. one that has rejuvenative effects, boosts memory, prevents cognitive deficits and improves brain function (Kapoor 1990; Nadkarni 1976; Shinomol and Muralidhara 2011).

Table 1 Common names for *Centella asiatica* in different regions or languages of the world

Region/language	Names	References
Arabic	Artaniyal-hindi, zarnab	Nadkarni (1976), Kapoor (1990)
Chinese	Tungchian, luei gong gen, ji xue cao	Brinkhaus et al. (2000), USDA (2016)
English	Indian Pennywort, Asiatic pennywort, marsh pennywort, pennyweed, sheeprat, Indian water navelwort	USDA (2016), Brinkhaus et al. (2000), Newall et al. (1996)
French	Hydrocotyle Asiatique, écuelle d'eau	Brinkhaus et al. (2000), USDA (2016)
German	Indischer wassernabel, Asiatisches Sumpfpfennigkraut	Brinkhaus et al. (2000), USDA (2016)
India (language given in brackets after name)	Brahmi, mandukaparni, cheka parni (Sanskrit); khulakudi, brahma-manduki (Hindi); karivana, karinga, undri (Marathi); thulkuri, brahma-manduki (Bengali); karbrahmi (Gujarati); saraswathi aku, manduka, brahmakuraku (Telugu); vellarai, babassa (Tamil); kutakam (Malayalam); ekpanni (Konkani)	Nadkarni (1976)
Indonesia	Kaki kuda, pegagan, anantan gede, gagan-gagan, gang-gagan, kirok gatok, panegowan, rending, calingan rambat, kos tekosan, pagaga, tungke-tungke, papaiduh, pepiduh, piduh, puhe beta, kaki kuta, tete karo, tete kadho	Brinkhaus et al. (2000)
Italian	Idrocotyle	Brinkhaus et al. (2000)
Japan	Tsubo-kusa, tsubokura	Brinkhaus et al. (2000), Kapoor (1990)
Malagasy	Talapetraka	(Rahajanirina et al. 2012)
Malaysia	Dawoopungah-gah, pegaga	Nadkarni (1976), Maulidiani et al. (2014)
Mauritius	Bavilaaqua	Brinkhaus et al. (2000)
Myanmar	Minkhuabin	Nadkarni (1976)
Nepal	Kholachagya	Kapoor (1990)
Persian	Sard Turkistan	Kapoor (1990)
Spanish	Sombrerito, hierba de clavo	USDA (2016)
Sri Lanka (Sinhalese)	Gotu kola; hingolukola	Nadkarni (1976), Kapoor (1990)
Swedish	Sallatsspikblad	USDA (2016)
Thailand	Bau-bog	Botanica (2017)
Tibetan	Sin-mnar	Kapoor (1990)

Preparations

For rejuvenatory (“rasayana”) effects, the herb is traditionally administered orally as the fresh juice “swarasam” or prepared in clarified butter as a “gritham” (Nadkarni 1976). For “mental weakness and for improving memory”, powder of the dried leaves is also given with milk (Nadkarni 1976). For other uses, topical or oral preparations include dried leaf powder, poultices of the fresh leaves, leaf juice, or decoctions of the dried leaf (Long et al. 2012; Nadkarni 1976). In modern use as a dietary

supplement, *C. asiatica* is typically sold as capsules of the dried herb or a dried extract, or as an herbal tincture. Extracts containing standardized mixtures of the purified triterpenes, which have been used in clinical studies, include TTFCA (total triterpenic fraction of *Centella asiatica*) and TECA (titrated extract of *Centella asiatica*) (Brinkhaus et al. 2000). More recently developed commercial preparations include standardized mixtures of purified triterpenes from *C. asiatica*, e.g. CASTTM (*Centella asiatica* selected triterpenes) and CenteroxTM by Indena SpA (Milan, Italy), or crude extracts which are



Fig. 1 *Centella asiatica* plant, showing spade-like leaves, internode stolons and small flowers. *Photo credit* iStock.com/bdspn

standardized to their triterpene content, e.g. Cente-vita™ (Indena) and Centellin® (Sabinsa Corp, New Jersey USA).

The present review will focus on the phytochemistry of *C. asiatica*, and the neuroprotective and cognitive enhancing properties of *C. asiatica* and its constituent compounds. Preclinical studies have used crude solvent extracts of the herb, or purified compounds, while clinical studies have used a plethora of products, often not well described (Tables 4a, b). A significant body of scientific research also exists on the wound healing and gastroprotective properties of preparations of *C. asiatica* or its triterpenes. Those studies have been reviewed elsewhere (Brinkhaus et al. 2000) and are outside the scope of the present review.

Phytochemistry

Major phytochemicals

An early comprehensive overview of secondary metabolites found in *C. asiatica* has been provided by Brinkhaus et al. (2000). These secondary metabolites are found in the aerial parts of the plants as well as in the roots and rhizomes (Brinkhaus et al. 2000). The *C. asiatica* leaf transcriptome was recently published and provides a detailed map of the genes involved in the synthesis of secondary metabolites (Sangwan et al.

2013). A Cytoscape network of 57 secondary metabolites typically found in *C. asiatica* (Table 2; Fig. 2) were clustered according to chemical 2D structure similarity using the Tanimoto method (Flowers 1998). Compounds of the isoprenoid pathway fall into four clusters: sesquiterpene, plant sterols, pentacyclic triterpenoids and saponins. Compounds that share the phenylpropanoid scaffold fall into three clusters: eugenol derivatives, caffeoylquinic acids (with sub-clusters of mono- or di-caffeoylquinic acids), and flavonoids (Fig. 2).

Considerable variation has been reported in the volatile constituents of essential oil preparations of *C. asiatica*. Essential oil from the aerial parts of *C. asiatica* contains high levels of sesquiterpenes (C_{15}) and monoterpenoids (C_{10}), including α -humulene, β -caryophyllene, bicyclogermacrene, germacrene-D, and myrcene as the major constituents (Oyedeji and Afolayan 2005; Wong and Tan 1994). Other constituents have been characterized as oxygenated sesquiterpenes (e.g. humulene epoxide ($C_{15}H_{24}O$), caryophyllene oxide ($C_{15}H_{24}O$)), oxygenated monoterpenes (e.g. menthone ($C_{10}H_{18}O$), α -terpineol ($C_{10}H_{18}O$)) and a sulfide sesquiterpenoid (mintsulfide, $C_{15}H_{24}S$) (Apichartsrangkoon et al. 2009; Oyedeji and Afolayan 2005). Essential oil extracts show antibacterial activities against Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella sonnei*) (Oyedeji and Afolayan 2005).

Table 2 Compounds present in *Centella asiatica*

Compound	CID (PubChem)	CAS (PubChem)	References
alpha-Humulene	5281520	6753-98-6	Oyedeki and Afolayan (2005)
Arjunolic acid	73641	465-00-9	Azerad (2016)
Asiatic acid	119034	464-92-6	Brinkhaus et al. (2000), Jamil et al. (2007)
Asiaticoside	108062	16830-15-2	Brinkhaus et al. (2000)
Asiaticoside B	91618002	125265-68-1	Brinkhaus et al. (2000)
Asiaticoside C	101103169		James and Dubery (2009)
Asiaticoside D	102212084		Azerad (2016)
Asiaticoside E	102212085		Azerad (2016)
Asiaticoside F	53317001		Azerad (2016)
Asiaticoside G	53320941		Azerad (2016)
β-Caryophyllene	5281515	87-44-5	Oyedeki and Afolayan (2005)
Bicyclogermacrene	5315347		Oyedeki and Afolayan (2005)
Brahminoside B	101504860		James and Dubery (2009)
Campesterol	173183	474-62-4	Jamil et al. (2007)
Castillicetin	102394640		Chandrika and Prasad Kumarab (2015)
Castilliferol	10526707		Chandrika and Prasad Kumarab (2015)
Catechin	9064	154-23-4	Mustafa et al. (2010)
Centellasapogenol A	15508087		Azerad (2016)
Centellasaponin A	21633803		Azerad (2016)
Centellasaponin B	101140416		Azerad (2016)
Centellasaponin C	101103167		Azerad (2016)
Centellasaponin D	101103168		Azerad (2016)
Centelloside E	101568838		Azerad (2016)
Centelloside D	56964357		Azerad (2016)
Chavicol	68148	501-92-8	Brinkhaus (et al. 2000)
Chebuloside II	102005082		Azerad (2016)
Chlorogenic acid			
(3-O-Caffeoylquinic acid)	1794427	327-97-9	Long et al. (2012)
Corosolic acid	6918774	4547-24-4	Yoshida et al. (2005)
Cryptochlorogenic Acid, (4-O-Caffeoylquinic acid)	9798666	905-99-7	Long et al. (2012)
1,3-Dicaffeoylquinic acid	6474640	19870-46-3	Long et al. (2012)
1,5-Dicaffeoylquinic acid	122685	30964-13-7	Long et al. (2012)
3,4-Dicaffeoylquinic acid (Isochlorogenic acid B)	5281780	14534-61-3	Long et al. (2012)
3,5-Dicaffeoylquinic acid (Isochlorogenic acid A)	6474310	2450-53-5	Long et al. (2012)
4,5-Dicaffeoylquinic acid (Isochlorogenic acid C)	6474309	32451-88-0	Long et al. (2012)
Epicatechin	72276	490-46-0	Mustafa et al. (2010)
3-Epimaslinic acid	25564831	26563-68-8	Jamil et al. (2007), Yoshida et al. (2005)
Eugenol acetate	7136	93-28-7	Brinkhaus et al. (2000)
Germacrene B	5281519		Oyedeki and Afolayan (2005)
Kaempferol	5280863	520-18-3	Devkota et al. (2010)
Madecassic acid, brahmlic acid	73412	18449-41-7	Brinkhaus et al. (2000)

Table 2 continued

Compound	CID (PubChem)	CAS (PubChem)	References
Madecassoside	45356919	34540-22-2	Brinkhaus et al. (2000)
Methyleugenol	7127	93-15-2	Brinkhaus et al. (2000)
Myrcene	31253	123-35-3	Brinkhaus et al. (2000), Oyedeji and Afolayan (2005)
Myricetin	5281672	529-44-2	Chandrika and Prasad Kumarab (2015)
Naringin	442428	10236-47-2	Sangwan et al. (2013)
Neochlorogenic acid (5-O-Dicaffeoylquinic acid)	5280633	906-33-2	Long et al. (2012)
Patuletin	5281678	519-96-0	Chandrika and Prasad Kumarab (2015)
Pomolic Acid	382831	13849-91-7	Yoshida et al. (2005)
Quadranside IV	10372074		Azerad (2016)
Quercetin	5280343	117-39-5	Sangwan et al. (2013)
Rutin	5280805	153-18-4	Sangwan et al. (2013)
Scheffuroside B	102212086		Azerad (2016)
Scheffuroside F	101675278		Azerad (2016)
Sitosterol	222284	68555-08-8	Yoshida et al. (2005)
Stigmasterol	5280794	83-48-7	Chandrika and Prasad Kumarab (2015)
Terminolic acid	12314613	564-13-6	Brinkhaus et al. (2000)
Ursolic acid	64945	77-52-1	Yoshida et al. (2005)

Centella asiatica is most known for its high content of pentacyclic triterpenoids (C₃₀), collectively referred also as “centelloids”. The structural diversity of pentacyclic triterpenoids found in *C. asiatica* has been reviewed previously (Azerad 2016; James and Dubery 2009). The centelloids can be grouped into two structural sub-types (Fig. 3), the ursane and the oleanane series, that differ by the methyl substitution pattern on C-19 and C-20 (Azerad 2016). Glycosylation of the saponinins usually involves the carboxylic acid group at C-28 (Azerad 2016).

The saponins, asiaticoside and madecassoside, and their aglycones, asiatic acid and madecassic acids, are the most abundant pentacyclic triterpenoids in *C. asiatica*. Saponins account for up to 8% of the dry mass of the herb (James and Dubery 2011). The levels of saponins and saponinins vary widely depending on the geographical origin, genetic, environmental and growth conditions (see “Chemical variation in *Centella asiatica*” section). Saponins are synthesized via the isoprenoid pathway. All isoprenoid biosynthetic pathways start with the isomeric 5-carbon precursors, isopentenyl diphosphate and dimethylallyl diphosphate. Assembly of three of these C₅ precursors by prenyl transferases yields farnesyl diphosphate (C₁₅).

Head-to-head condensation of two molecules farnesyl diphosphate catalyzed by squalene synthase yields squalene (C₃₀). Squalene is converted by squalene epoxidase in the presence of O₂ and NADPH to (3S)-squalene 2,3-epoxide (2,3-oxidosqualene), the common precursor of the biosynthetic pathways that lead to sterols and triterpenoids, respectively. In both biosynthetic pathways the cyclization of 2,3-oxidosqualene is facilitated by a family of 2,3-oxidosqualene cyclases. However, dependent on the conformation adopted by 2,3-oxidosqualene during cyclization, the dammarenyl cation is formed leading to triterpenoids of *C. asiatica* via the action of specific 2,3-oxidosqualene cyclase, α -amyrin synthase, while sterols are formed via protosteryl cation and the action of cyclo-artenol synthase (Azerad 2016; Gallego et al. 2014; Mangas et al. 2008; Muller et al. 2013; Tugizimana et al. 2015).

C. asiatica is also rich in chlorogenic acids, a diverse group of compounds formed by quinic acid esterified to cinnamic acid derivatives (Ncube et al. 2016). The enzyme phenylalanine ammonia lyase catalyzes the E2 elimination of ammonia from L-phenylalanine to produce *trans*-cinnamic acid, which is further transformed to 4-hydroxycinnamic acid (*p*-

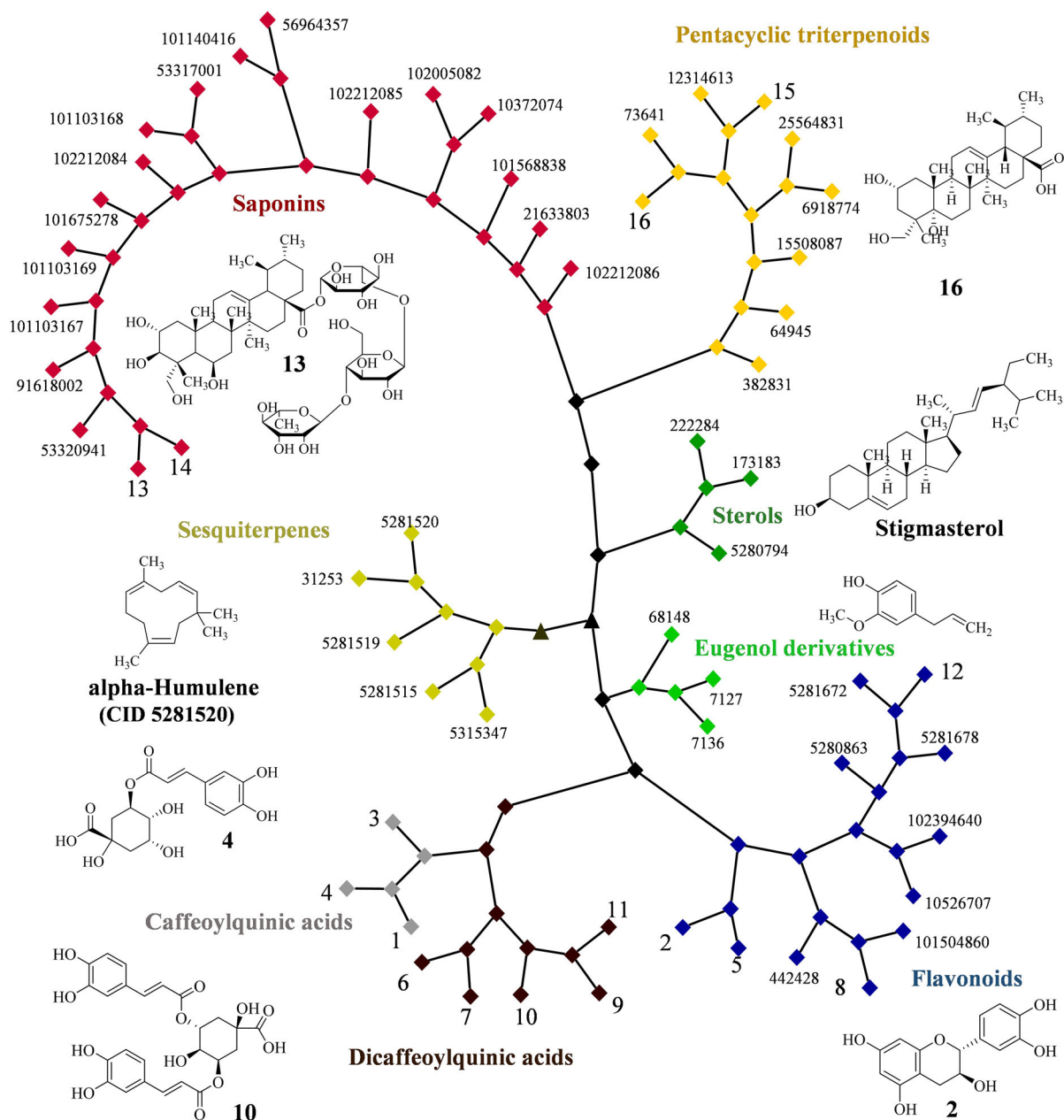


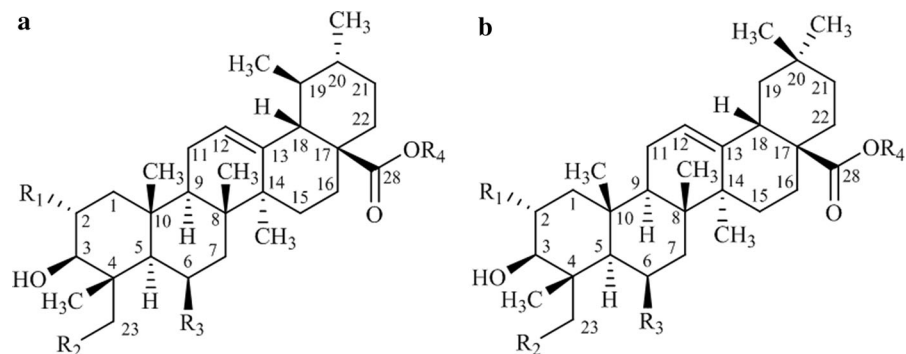
Fig. 2 Cytoscape network for compounds found in *C. asiatica*. The clustering relationship is based on structural similarities among 57 compounds using the Tanimoto algorithm. Seven main clusters are present: saponins, pentacyclic triterpenoids, sterols, sesquiterpenes, eugenol derivatives, caffeoylquinic acids (subclusters of mono- or di-caffeoylquinic acids) and

flavonoids. A representative compound of each cluster is shown. The compound numbers displayed correspond to the PubChem CIDs (Table 2), and the compound numbers 1–16 refer to the compounds given in Fig. 4. Filled triangle indicates a node with a Tanimoto score of < 0.68. A Tanimoto score > 0.68 is statistically significant at the 95% CI (Kim et al. 2012b)

coumaric acid) via the P450-dependent enzyme 4-cinnamic acid hydroxylase. Additional enzymatic hydroxylation and methylation reactions yield hydroxycinnamic acid derivatives e.g. caffeic acid with the

typical *ortho* oxygenation pattern. Esterification of hydroxyl groups on L-quinic acid with the carboxyl function of caffeic acid yields caffeoylquinic acids (CQAs). For instance, the ester of caffeoyl acid

Fig. 3 Structural sub-types of centellosides: **a** ursane family and **b** oleanane family. $R_1 - R_3 = \text{OH}$ or H , $R_4 = \text{H}$ or oligosaccharide. Adapted from Azerad (2016)



involving the 3-hydroxyl position of L-quinic acid yields 3-*O*-caffeoylquinic acid, also referred as chlorogenic acid. Structural isomers of caffeoyl esters involve other hydroxyl sites on the quinic acid: 4-*O*-caffeoylquinic acid (4-CQA or cryptochlorogenic acid) and 5-*O*-caffeoylquinic acid (5-CQA or neochlorogenic acid). Caffeoylquinic acids undergo positional isomerization in aqueous solution (Xie et al. 2011). Thus, in case of extraction protocols based on water or aqueous solvent mixtures the content of the different caffeoylquinic acids may not reflect the *de facto* contents of the different caffeoylquinic acids in the plant (Xie et al. 2011).

In *C. asiatica*, the following isomeric dicaffeoyl esters are commonly found: 1,3-dicaffeoylquinic acid, 1,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid (Gray et al. 2014). Some dicaffeoylquinic acids are also referred to by the trivial names isochlorogenic acid A, B or C (Table 2). Irbic acid (3,5-*O*-dicaffeoyl-4-*O*-malonylquinic acid) is a dicaffeoylquinic acid derivative with a malonyl group on the quinic acid moiety, which has been reported in *C. asiatica* species (Maulidiani et al. 2014) and cell cultures (Antognoni et al. 2011).

C. asiatica contains many phenolic constituents, including flavonoids, such as catechin, epicatechin, kaempferol, quercetin and related glycosides (Ncube et al. 2016). The biosynthesis of flavonoids combines the shikimate pathway with the acetate pathway using *p*-cinnamoyl-CoA as the starter unit and three malonyl-CoA units for chain extension. Chalcone synthase forms naringenin-chalcone via a Claisen-type reaction. An intramolecular Michael-type nucleophilic attack yields the A ring of the flavanone, naringenin. Further enzymatic transformation of

naringenin leads to a range of flavones (e.g. apigenin) and flavonols, e.g. kaempferol and quercetin (Dewick 2002; Muller et al. 2013; Ncube et al. 2016; Winkel-Shirley 2001). Flavonoids esterified to hydroxycinnamic derivatives, such as castilliferol (kaempferol-3-*p*-coumarate) and castillicetin (quercetin-3-caffeate), have been isolated from the aqueous methanol extracts of *C. asiatica* (Subban et al. 2008).

Contemporary methods for the characterization and fingerprinting of *C. asiatica* extracts

Traditionally, the analysis of *Centella* plant extracts was based on thin layer chromatography (Bonfill et al. 2006; Brinkhaus et al. 2000). From as early as 1996, the characterization and finger printing of *Centella* plant extracts have been mostly based on HPLC methods (Gunther and Wagner 1996). HPLC with ultraviolet (UV) detection is commonly employed for the characterization and quantification of flavonoids, mono- and dicaffeoylquinic acids and saponins. Due to the lack of strong UV-absorbing chromophores in centellosides, HPLC methods in conjunction with evaporative light scattering detection have been developed for the separation, isolation and characterization of centellosides (Zhang et al. 2008). High-speed countercurrent chromatographic techniques have been described for the efficient isolation of larger amounts of centellosides (Diallo et al. 1991; Du et al. 2004). For the structural identification of natural products, 1- and 2-dimensional Nuclear Magnetic Resonance Spectroscopy (NMR) techniques remain superior. Examples in which NMR techniques were used for the identification of centellosides are available (Antognoni et al. 2011; Subban et al. 2008).

The emergence of chromatographic techniques hyphenated to mass spectrometry (MS) techniques has gained popularity for the analysis of plant extracts in general. Modern mass spectrometry platforms offer accurate mass measurements in combination with collision-induced dissociation techniques for structural analysis and quantification of compounds in complex mixtures. LC–MS/MS-based studies of *Centella* extracts with detailed descriptions of experimental conditions are available (Gray et al. 2014; Jiang et al. 2016; Maulidiani et al. 2014).

In our laboratory, we favor the use of ultra-performance liquid chromatography (UPLC) in conjunction with accurate mass high-resolution tandem mass spectrometry for the chemical analysis and characterization of plant extracts. For instance, our recent work on the bioactivity of *C. asiatica* constituents uses aqueous extracts of the aerial parts of the plant obtained after boiling under reflux for 2 h (Gray et al. 2014). These aqueous extracts contain high levels of chlorogenic acids and dicaffeoylquinic acids besides minor levels of pentacyclic triterpenoids and flavonoids. Compounds present in this type of extract are shown in Fig. 4 and numbered according to their elution time (experimental conditions described in Fig. 5).

A typical UPLC–MS chromatogram of an aqueous extract of *C. asiatica* is shown in Fig. 5. Ethanol extracts of *C. asiatica* differ markedly from the aqueous extracts in that they contain high levels of asiaticoside and madecassosides, and their corresponding aglycones, asiatic acid and madecassic acids (Fig. 6). This example illustrates that standardization and detailed characterization of *C. asiatica* extracts are necessary to reliably and reproducibly study the biological activity of *C. asiatica* preparations.

Although isomeric compounds cannot be resolved solely by mass, some of them can be differentiated by their relative intensity of fragment ions in the mass spectra. This is the case for mono-caffeoylquinic acids using collision induced dissociation (CID) fragmentation in the negative ionization mode (Fig. 7). Five major products were observed m/z 191.0599, [quinic acid–H][−]; m/z 179.0376, [caffeic acid–H][−]; m/z 173.0518, [quinic acid –H₂O –H][−]; m/z 135.0484 [caffeic acid –CO₂–H][−]; and m/z 93.0385 [phenol–H][−]. As described by Xie et al. (2011), 3-*O*-CQA can be distinguished from 4-*O*-CQA by its base peak with m/z 191.0599, whereas the base peak of 4-*O*-CQA

appears at m/z 173.0518 (Fig. 7). Similarly, Clifford et al. (2005) showed that dicaffeoyl quinic acid isomers can be discriminated using CID fragmentation. In cases where isomers cannot be distinguished by their fragmentation behavior, their chromatographic retention behavior may allow discrimination between isomers. For instance, the structural isomers of isochlorogenic acids have the same accurate mass and elemental composition but differ in their retention behavior during reversed-phase chromatography (compounds 9, 10, 11 in Figs. 5, 6).

Ion mobility spectrometry (IMS) in conjunction with mass spectrometry has emerged as an additional tool for the detection and characterization of secondary metabolites in plant extracts and tissues. The addition of ion mobility spectrometry increases peak capacity on a millisecond timescale and provides insight into gas-phase ion structures. In the drift region of the ion mobility device, an ion moves according to its charge, size, shape and average collisional cross-section (between the ion and a background gas). In our laboratory we use travelling wave ion mobility spectrometry (TWIMS) coupled to a high-resolution time-of-flight (TOF) analyzer, a Waters SYNAPT G2 HDMS (Waters Corp., MA, USA), for the analysis of complex biological matrices. This instrument configuration features a TWIMS cell between two collision cells, the T-wave trap (pre-IMS) and transfer cell (post-IMS). For the detection and characterization of secondary metabolites in aqueous extracts of *C. asiatica* we use UPLC in conjunction with electrospray ionization and the MS^E acquisition mode. The MS^E acquisition mode is a data independent acquisition technique with provides exact mass information by working in the low-energy regime (precursor MS) and fragment ion information in the high-energy regime for every detectable analyte. Figure 8 shows a two-dimensional map visualizing drift time *versus* retention time of an aqueous extract of *C. asiatica*. The combination of UPLC separation, nested gas phase mobility separation of ions and accurate mass measurement allows comprehensive detection of the constituents within a *C. asiatica* extract. In addition, fragmentation post ion mobility separation provides fragment ion information. In this mode, ions are separated in the T-wave ion mobility cell and subsequently subjected to collision-induced fragmentation in the transfer region. Applying collision energy to the transfer device (collision energy ramp from 20 to 65 V)

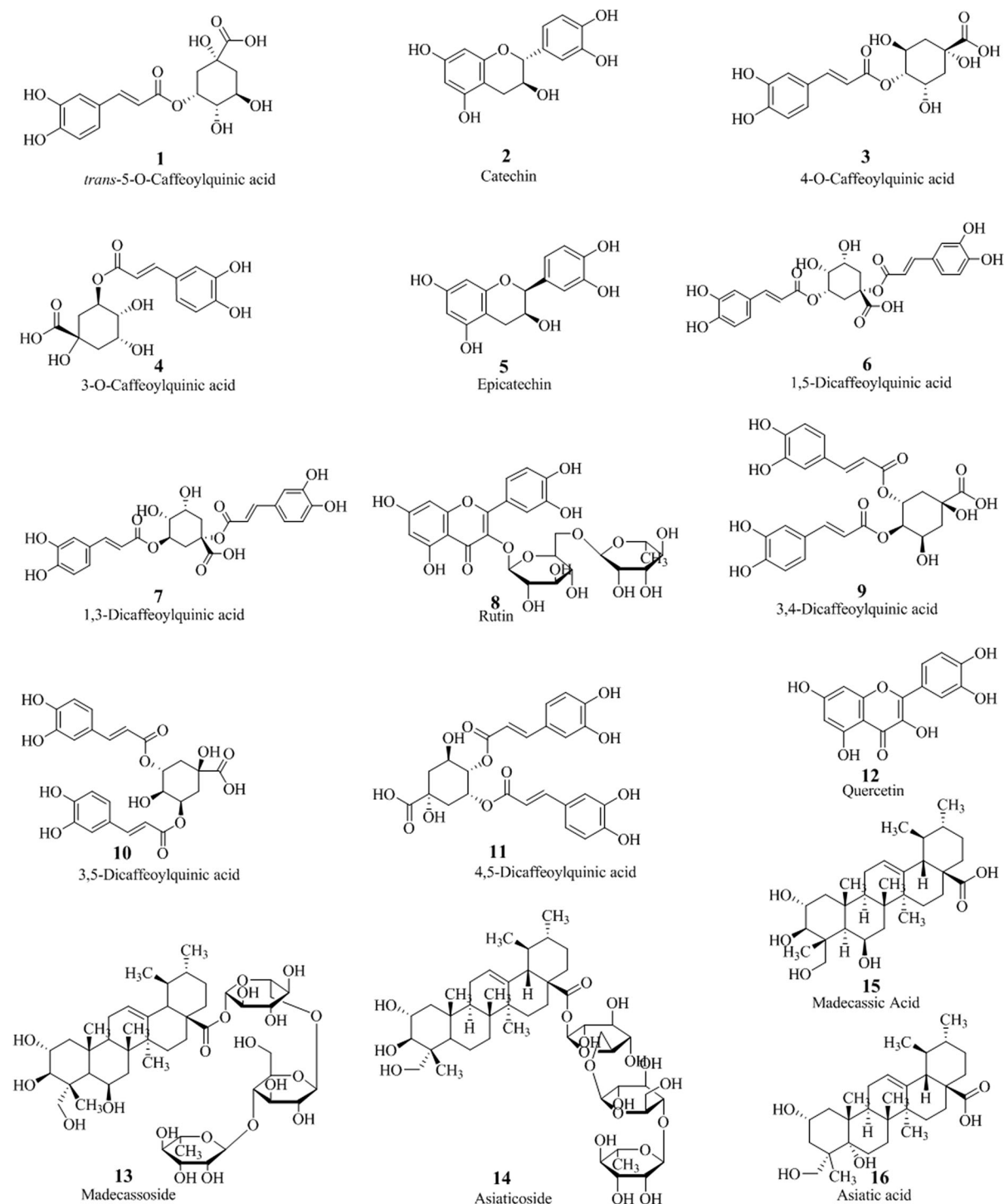


Fig. 4 Structures of compounds detected in aqueous extracts of *C. asiatica*

causes fragmentation of the mobility-separated precursor ions. Because the fragment ions preserve their velocity of the precursor ion, the fragment ions align

with the drift times of the precursor ions. As an example of the use of this technique, asiaticoside ($[M-H]^-$ m/z 957.5054) and madecassoside ($[M-H]^-$ m/z 973.5003),

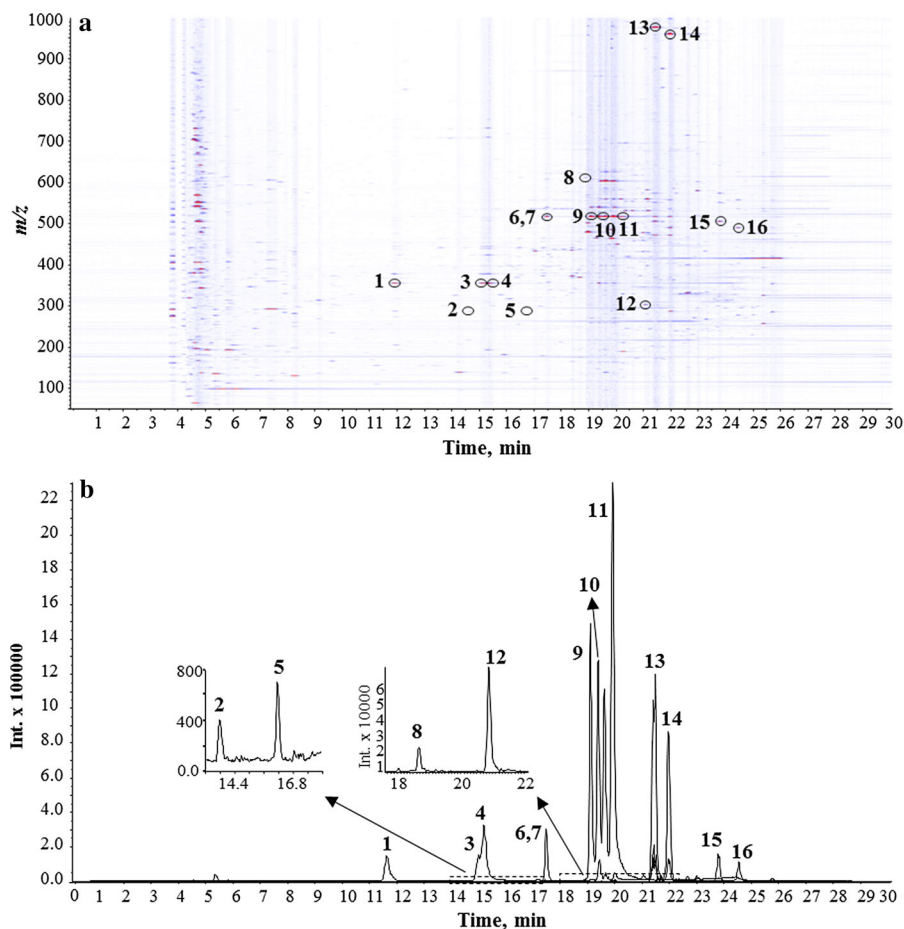


Fig. 5 UPLC-MS analysis of an aqueous extract of *C. asiatica*, 10 μ l injection (5 mg/ml of dry mass resuspended in 70% aqueous methanol). **a** Ion intensity map (of an aqueous extract of *C. asiatica*). In this 2D visualization, retention time is given on the x-axis, the m/z values are shown on the y-axis, and colors represents signal intensity. **b** Extracted ion chromatograms for 16 commonly found compounds. [$M-H$] $^-$ (**1**, **3**, **4** m/z 353.09; **2**, **5** m/z 289.07; **6**, **7**, **9–11** m/z 515.12; **8** m/z 609.15; **12** m/z 301.03; **13** m/z 973.50; **14** m/z 957.51; **15** m/z 503.34; **16** m/z 487.34). Chromatographic separation was conducted using a Shimadzu Nexera UPLC system equipped with Inertsil Phenyl-3 column (150 \times 4.6 mm, 5 μ m). Mobile phase A was water with 0.1% formic acid, and mobile phase B was methanol with 0.1% formic acid. The gradient started with 5% B and was held for

1 min, followed by a 10 min linear gradient from 5% to 30%. The gradient was then stepped to 100% B at 23 min and held for 12 min and finally, stepped back to 5% B to equilibrate the column. The flow rate was 0.4 ml/min, and the column temperature was maintained at 45 $^\circ$ C. An AB Sciex Triple TOF 5600 mass spectrometer equipped with a TurboSpray electrospray ionization source operated in the negative ionization mode was used. The instrument was operated in the information-dependent acquisition (IDA) mode using a collision energy of 40 V. Compound identity is based on accurate mass, isotopic pattern, retention time, MS/MS spectra and standard addition using authentic compounds. Structures of compounds **1** to **16** are given in Fig. 4. (Color figure online)

both present at low levels in the aqueous extract of *C. asiatica* (Gray et al. 2014), were subjected to fragmentation in the transfer region by working in the high energy regime. Both compounds produce their respective deprotonated saponinins [madecassic acid—H] $^-$ (m/z 503.3373) and [asiatic acid—H] $^-$ (m/z 487.3419). In addition, both saponins generate a fragment ion with m/z 469.1592 attributed to the deprotonated

oligosaccharide moiety. Drift time distributions for the fragment ions and precursor ions, proposed transfer dissociation, and fragmentation spectra are shown in Fig. 8.

In depth characterization and finger printing studies of *C. asiatica* extracts and preparations have yet to become available; however, applications of IMS-MS focusing on the characterization of isomeric

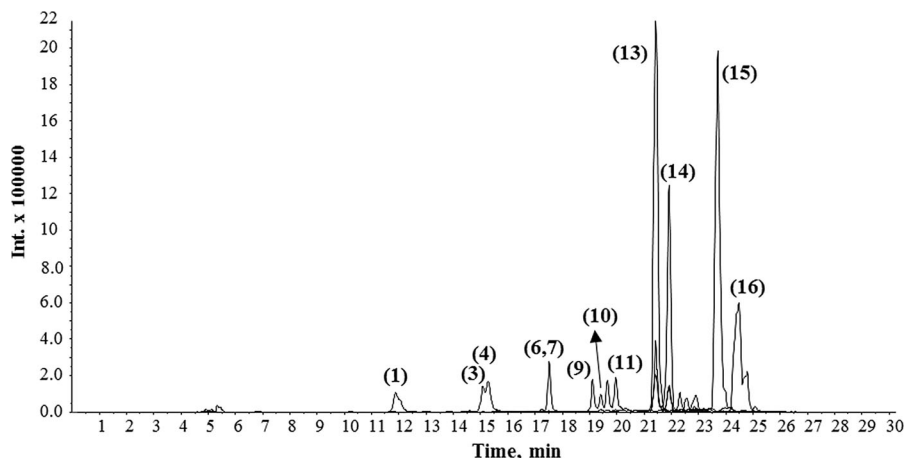


Fig. 6 UPLC-MS chromatogram of an ethanolic extract of *C. asiatica*, 10 μ l injection (5 mg/ml of dry mass resuspended in methanol 70%). Shown is an overlay of extracted ion chromatograms for commonly found compounds. $[M-H]^-$ (1,

3, 4 m/z 353.09; 6, 7, 9–11 m/z 515.12; 8 m/z 609.15; 12 m/z 301.03; 13 m/z 973.50; 14 m/z 957.51; 15 m/z 503.34; 16 m/z 487.34). Experimental conditions as described in the legend of Fig. 5. Structures of compounds 1–16 are given in Fig. 4

compounds that are also found in *C. asiatica* are available. For example, IMS-MS/MS studies have been reported for the elucidation of structural isomers of chlorogenic acids (Shinomol and Muralidhara 2011) and carotenoids (Dong et al. 2010). The combination of ion mobility spectrometry and high-resolution mass spectrometry adds selectivity and specificity to the analysis of plant metabolites by increasing the separation space for complex plant extracts and matrices (Pacini et al. 2015; Stark et al. 2013) enabling the separation of isomeric metabolites and improving structure elucidation of complex natural products. The availability of commercial instruments that combine ion mobility separation with mass spectrometry will likely advance the characterization and standardization efforts of plant extract and traditional herbal medicine preparations.

Emerging analytical trends include the application of mass spectrometry-driven metabolomic studies of extracts of *Centella* species and cell cultures after perturbations. For instance, gas chromatography (GC)-MS and LC-MS-based chemometric studies of extracts of *C. asiatica* cultures have been used to investigate the effect of methyl jasmonate on metabolite composition (Tugizimana et al. 2015). Similarly, a LC-MS/MS-driven method in combination with multivariate data analysis has recently been employed for determining the effect of salicylic acid on the profile of chlorogenic acids in *C. asiatica* cultures (Ncube et al.

2016). The advent of commercially available IMS-MS systems has led to exciting applications in the field of plant metabolomics (Maldini et al. 2015; Zhang et al. 2014); however, IMS-MS-based studies of *C. asiatica* preparations or cell cultures are lacking.

Chemical variation in *Centella asiatica*

The increasing commercial importance of *C. asiatica* as a source of bioactive extracts and isolated triterpenoids has led to a search for elite chemotypic strains of the herb, as well as means of optimizing the yield of the active compounds from a given strain. There have been several studies in different growing regions of *C. asiatica* comparing the levels of the triterpenoid and phenolic compounds in multiple accessions of the herb. In eleven *C. asiatica* accessions collected from the Nilgiri hill region in India, wide variations were seen in the content of asiatic acid (0.04–0.58%), asiaticoside (0.11–1.56%) and madecassoside (0.29–6.09%) (Srivastava et al. 2014). A comparison of 14 phenotypically distinct *C. asiatica* accessions collected from an unspecified region of India again showed wide variability in these triterpenes as well as madecassic acid, with total content of these four compounds varying from 0.26 to 11 mg/g fresh herb weight (Singh et al. 2015). Twelve *C. asiatica* accessions collected from multiple regions of South Africa had asiaticoside levels ranging from 0.75 to

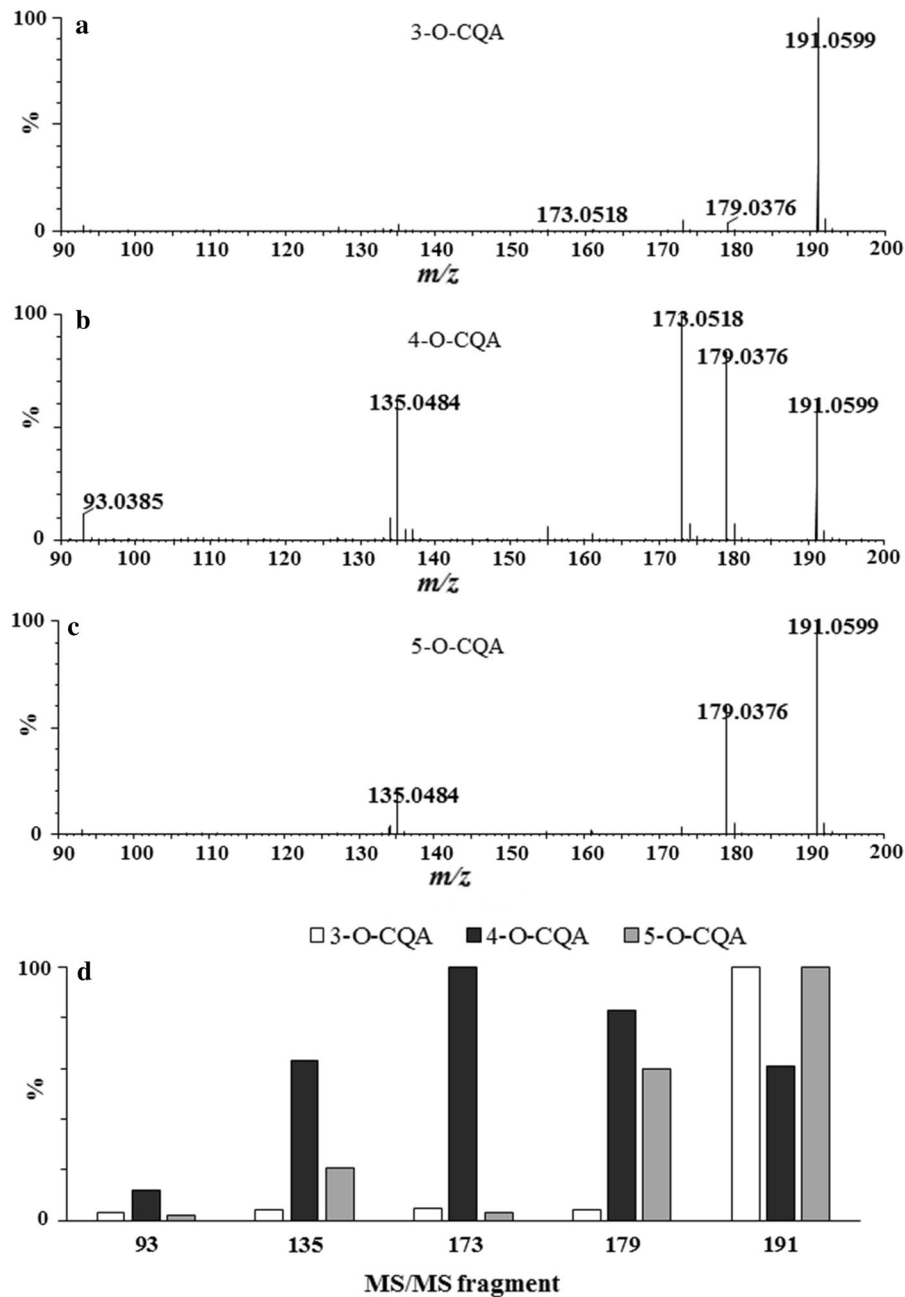


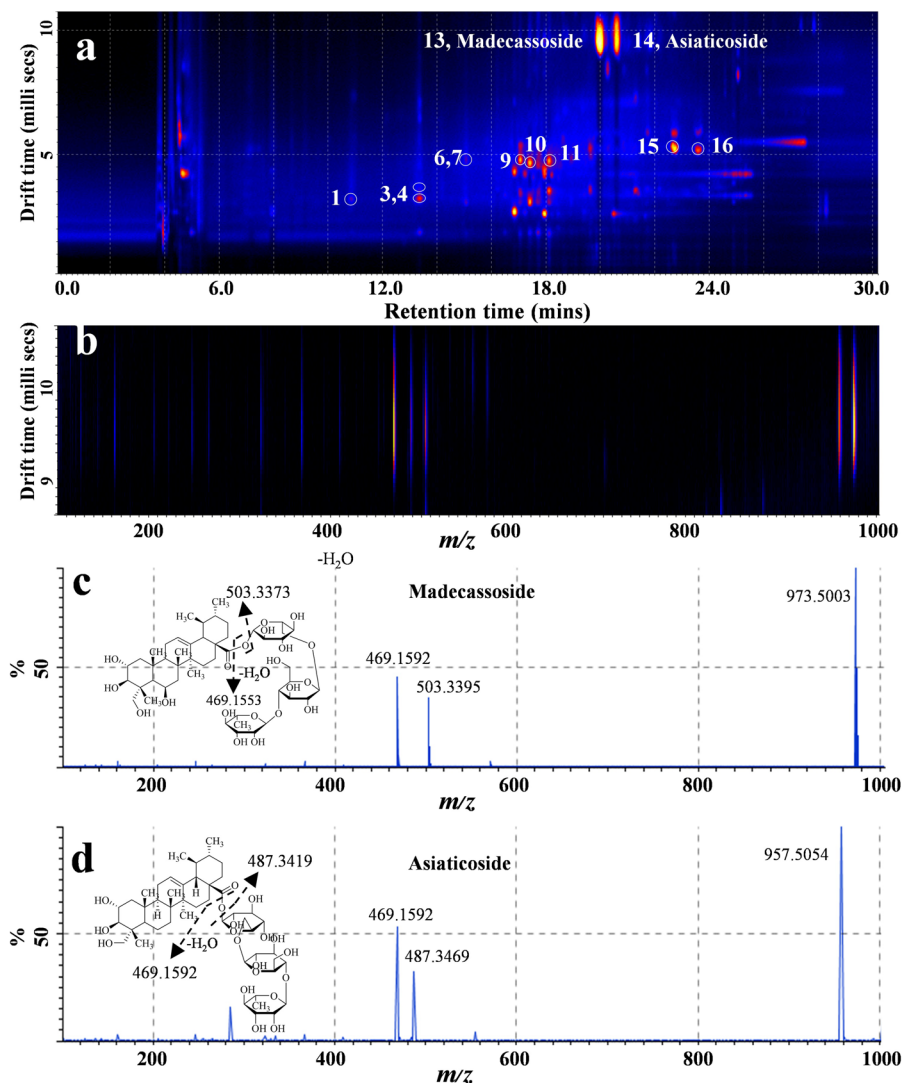
Fig. 7 Mass spectra of isomeric mono-caffeoylquinic acids recorded in negative ionization mode showing differences in the intensity of MS fragment ions. Standards were injected by infusion (10 μ l/min in methanol:water 70:30 V/V, 1 mg/l). A Synapt G2 HDMS (Waters Corp., MA, USA) was used for ion

detection. The cone voltage was 20 V and the transfer energy was set at 25 eV. **a** 3-O-caffeoylquinic acid, **b** 4-O-caffeoylquinic acid, **c** 5-O-caffeoylquinic acid, **d** Intensity comparison for major MS/MS product ions of CQAs. Data are in accord with Xie et al. (2011)

2.06% dry weight (Long et al. 2012). A study in Madagascar compared triterpene levels in two *C. asiatica* morphotypes, reniform leaves (type A;

$n = 32$ collection sites) and round leaves (type B; $n = 13$ collection sites), from the Eastern and Western regions of the island respectively (Rahajanirina et al.

Fig. 8 ESI q-IMS-MS/MS analysis of an aqueous extract of *C. asiatica* (5 mg/ml of dry mass resuspended in methanol 70%, 10 μ l injection). **a** 2D map visualization of drift time versus retention time, **b** drift time distribution of ions, **c**, **d** fragmentation spectrum and proposed transfer dissociation of madecassoside ($[M-H]^-$ m/z 973.5) and asiaticoside ($[M-H]^-$ m/z 957.5), respectively. *Method* Acquity UPLC chromatographic conditions were as described in Fig. 5. The LC system was coupled to a Synapt G2 HDMS (Waters Corp., MA, USA) used for detection in negative ion electrospray mode. Nitrogen was used as carrier gas for ion mobility experiments. Data acquisition range was m/z 50–1200. The cone voltage was 20 V. The T-wave ion mobility cell was operated at 800 m/s and the wave amplitude was set at 35 V. Helium and nitrogen IMS carrier were both set at 80 ml/min. (Color figure online)



2012). Type A plants were found to have significantly higher levels of asiaticoside (3.7 vs 2%) and madecassoside (3.6 vs 2%) than the Type B plants, although aglycone levels did not differ significantly. In addition to the triterpenoids, large differences in total flavonoid content (18, 30 and 144 mg/g) have been observed in three accessions from the Assam region of India, although antioxidant activity and total phenolics did not differ as widely (Upadhyaya and Saikia 2012).

Large seasonal variations have been documented in levels of triterpenes, chlorogenic acids and the flavonoid kaempferol in *C. asiatica* plants growing in Australia (Alqahtani et al. 2015), in agreement with seasonal effects on triterpenes reported in Madagascar (Rahajanirina et al. 2012). Light and drying conditions

have also been shown to affect triterpene levels (Srithongkul et al. 2011). Plants grown with full daylight exposure had higher levels of triterpenes, flavonoids chlorogenic acids and radical scavenging activity than those grown in 50% shade (Maulidiani et al. 2012). Plants dried at lower temperatures had higher yields of triterpene saponins than those dried at higher temperatures (Plengmuankhae and Tantitadapitak 2015).

Comparison of *C. asiatica* accessions obtained from diverse parts of India including the Andaman Islands, and propagated under identical conditions, has shown that the triterpenoid saponin content is a highly heritable trait (Lal et al. 2017), and elite strains can be identified and cultivated (Thomas et al. 2010). Zhang

et al. (2009) have compared LC-UV fingerprints of extracts from 14 populations of *C. asiatica* collected from different regions in China (Zhang et al. 2009). Hierarchical clustering analysis of 12 common peaks (of which only three were identified as asiatic acid, asiaticoside and madecassoside) from plant extracts showed the existence of four distinct chemotypes of *C. asiatica*. A subsequent study in 2012 examined inter-simple sequence repeat (ISSR) markers of 162 individuals from these 14 populations, and found high genetic diversity across the species, with greater diversity in populations from some regions than others (Zhang et al. 2012b). Interestingly, the 14 populations clustered into four genetic groups which were similar to the four chemical groups found by chemical clustering in the earlier study (Zhang et al. 2009)

These studies demonstrate that genetic and geographical factors as well as cultivation, harvesting and processing practices all influence the chemical profile of *C. asiatica*. While this has been best researched for the triterpene compounds, the other potentially active components of *C. asiatica*, such as flavonoids and chlorogenic acid derivatives, are also clearly influenced by these factors. These reports highlight the importance of evaluating the chemical characteristics of *C. asiatica* material used in scientific studies, as well as clear reporting in publications of the geographical source, method of preparation and chemical features of the materials studied. This would not only ensure that the materials tested contained active compounds, but also allow for better comparison between studies.

Neurological activities of *Centella asiatica* and its constituents

The observed effects of *C. asiatica* extracts and constituents in preclinical neurological systems are summarized in Table 3.

Anxiolytic, anti-convulsant, anti-ischemic injury effects of *C. asiatica*

C. asiatica has many reported beneficial neurobehavioral effects. Its anxiolytic properties have been demonstrated in healthy rodents (Wijeweera et al. 2006), as well as chronically stressed mice and mice

with sleep deprivation-induced anxiety (Chanana and Kumar 2016; Wanasuntronwong et al. 2012). In the sleep-deprived mice, *C. asiatica* treatment attenuated oxidative damage and decreased anxiety. The effect of the plant was enhanced when co-administered with nitric oxide antagonists, and decreased with nitric oxide agonists suggesting the possible involvement of nitric oxide modulating mechanisms in the anxiolytic effects (Chanana and Kumar 2016).

While early studies suggested the anxiolytic effects of *C. asiatica* may be related to more general sedative effects of the plant (Sakina and Dandiya 1990), recent studies have failed to confirm this (Wijeweera et al. 2006). Instead, new evidence suggests its anxiolytic effects may be due to effects on the GABA system. Aqueous and ethanolic extracts of *C. asiatica* have been shown to substantially increase the activity of glutamate decarboxylase, a key enzyme in GABA synthesis (Awad et al. 2007). Since GABA is an inhibitory neurotransmitter, these effects on GABA synthesis may also contribute to *C. asiatica*'s anti-convulsant properties.

C. asiatica treatment has been shown to be protective in rodent models of seizures (Gupta et al. 2003; Sakina and Dandiya 1990). It has been suggested that effects on the acetylcholine system could mediate its anti-convulsant activity. In a rat seizure model, butanol, ethyl acetate, and hexane extracts of *C. asiatica* all normalized acetylcholine levels and acetylcholinesterase activity in the brains of treated animals (Visweswari et al. 2010).

The neuroprotective effects of *C. asiatica* have also been demonstrated in rodent models of stroke. Oral treatment with an ethanol extract of the plant decreased infarct volume and improved neurobehavioral activity resulting from middle cerebral artery occlusion in rats (Tabassum et al. 2013). In this case, the neuroprotective effects were credited to the antioxidant effects of the extract, as the improvements were accompanied by decreased levels of reactive oxygen species (ROS) and markers of lipid peroxidation, and increased activity of several antioxidant enzymes. This finding was later confirmed by Doknark et al. (2014) who found that oral treatment with an ethanolic extract improved behavioral impairments and significantly reduced markers of oxidative damage in the brain (Doknark et al. 2014).

Table 3 Summary of the effects of *C. asiatica* and some of its constituent compounds in preclinical neurological systems

Test material	Neuroactive property	Potential mechanism
<i>Centella asiatica</i> extract	Cognitive enhancing in models of stroke, aging ^{1, 2} , Alzheimer's Disease ^{3, 4}	Decreased oxidative damage ^{1, 7–10, 27–32}
	Neuroprotective in models of A β toxicity ^{4–7} , Parkinson's disease ⁸ , Huntington's disease ^{9,10} , stroke ^{11, 12} , aluminum toxicity ¹³ and arsenic exposure ¹⁴	Increased NRF2 expression and activation ^{2, 31}
	Anxiolytic ^{15, 16, 17}	Increased mitochondrial enzyme activity ^{9, 31}
	Anti-convulsant ^{18, 19, 20}	Increased mitochondrial gene expression ^{2, 31}
	Neuritogenic ^{21–26}	Anti-inflammatory ³⁰
	Increased synaptogenesis ²	Increased glutamate decarboxylase activity ³³
		Inhibition of acetylcholinesterase ¹
		Restored calcium homeostasis ³¹
		Increased CREB phosphorylation ⁵
		Inhibition of PLA2 ^{34, 35}
Triterpenes	Cognitive enhancing in models of aging ^{36, 37} , stroke ³⁸ , VPA ³⁹ and glutamate-induced ⁴⁰ cognitive impairment	Decreased oxidative damage ⁴⁶
	Neuroprotective in models of A β toxicity ^{41, 42} , stroke ^{43–45} , spinal cord injury ⁴⁶ and ceramide toxicity ⁴⁷	Increased Nrf2 expression ⁴⁶
	Increased hippocampal cell proliferation ^{48, 49}	Increased histone deacetylase activity ³⁹
	Anxiolytic ^{15, 50}	Modulation of MAPK, PKA, CAM kinase and Notch signaling pathways ^{36, 38, 49}
	Increased synaptogenesis ^{36, 37}	Anti-inflammatory ^{38, 43, 44}
CQAs	Cognitive enhancing in models of stroke ^{51, 52} , aging ^{53, 54} and Alzheimer's disease ^{55–60}	Decreased oxidative damage ^{52, 55–58, 60}
	Neuroprotective in models of A β toxicity ^{31, 56} , stroke ⁶¹ , NMDA toxicity ⁶² and glutamate toxicity ^{56, 63}	Increased NRF2 expression and activation ^{31, 61, 64}
	Increased synaptogenesis ^{56, 58, 60}	Increased CREB phosphorylation ⁶²
		Restored calcium homeostasis ^{56, 63}
		Modulation of AKT, ERK, p38MAPK, GSK3B and JNK signaling pathways ^{56, 58, 60, 62}
		Anti-inflammatory ^{52, 56, 58, 60}
		Increased mitochondrial enzyme activity ³¹
		Increased mitochondrial gene expression ³¹
		Increased activity of glucose metabolizing enzymes ^{53, 54}
		Inhibition of acetylcholinesterase ^{55, 57, 59}
	Decreased 5-LPO expression ⁵²	

¹Kumar et al. (2011), ²Gray et al. (2016), ³Kumar et al. (2009), ⁴Soumyanath et al. (2012), ⁵Xu et al. (2008), ⁶Gray et al. (2014), ⁷Chen et al. (2015), ⁸Haleagrahara and Ponnusamy (2010), ⁹Shinomol and Muralidhara (2008b), ¹⁰Shinomol and Muralidhara (2008a), ¹¹Tabassum et al. (2013), ¹²Doknark et al. (2014), ¹³Flora and Gupta (2007), ¹⁴Prakash and Kumar (2013), ¹⁵Wijeweera et al. (2006), ¹⁶Wanasuntronwong et al. (2012), ¹⁷Chanana and Kumar (2016), ¹⁸Sakina and Dandiyi (1990), ¹⁹Gupta et al. (2003), ²⁰Visweswari et al. (2010), ²¹Soumyanath et al. (2005), ²²Mohandas Rao et al. (2006), ²³Gadahad et al. (2008), ²⁴Mohandas Rao et al. (2009), ²⁵Mohandas Rao et al. (2012), ²⁶Wanakhachornkrai et al. (2013), ²⁷Dhanasekaran et al. (2009), ²⁸Pittella et al. (2009), ²⁹Floridata (2016) and Meena et al. (2012), ³⁰Ramesh et al. (2014), ³¹Gray et al. (2015), ³²Kumari et al. (2016), ³³Awad et al. (2007), ³⁴Barbosa et al. (2008), ³⁵Defillipo et al. (2012), ³⁶Lin et al. (2013), ³⁷Lin et al. (2014), ³⁸Chen et al. (2014), ³⁹Umka Welbat et al. (2016), ⁴⁰Xu et al. (2012), ⁴¹Mook-Jung et al. (1999), ⁴²Jew et al. (2000), ⁴³Krishnamurthy et al. (2009), ⁴⁴Luo et al. (2014), ⁴⁵Sun et al. (2015), ⁴⁶Jiang et al. (2016), ⁴⁷Zhang et al. (2012a), ⁴⁸Nasir et al. (2011), ⁴⁹Sirichoat et al. (2015), ⁵⁰Ceremuga et al. (2015), ⁵¹Pinheiro Fernandes et al. (2014), ⁵²Liang et al. (2015), ⁵³Han et al. (2010), ⁵⁴Sasaki et al. (2013), ⁵⁵Kwon et al. (2010), ⁵⁶Kim et al. (2012a), ⁵⁷Choi et al. (2014), ⁵⁸Subash et al. (2015), ⁵⁹Deshmukh et al. (2016), ⁶⁰Wang et al. (2016), ⁶¹Cao et al. (2010), ⁶²Tian et al. (2015), ⁶³Mikami and Yamazawa (2015), ⁶⁴Boettler et al. (2010)

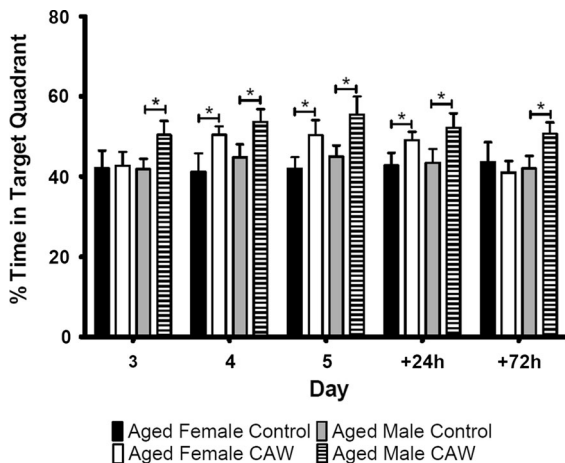


Fig. 9 Morris water maze probe data of healthy aged male and female mice treated with a water extract of *Centella asiatica* (CAW, 2 mg/ml dissolved in the drinking water) for 2 weeks prior to, and during testing. CAW increased memory retention in both male and female healthy aged mice. Extract treated animals spent significantly more time in the target quadrant than control, untreated mice. Adapted from Gray et al. *J. Ethnopharmacology* (2016)

Cognitive enhancing effects of *C. asiatica*

The cognitive enhancing properties of the plant have long-been recognized in the Ayurvedic tradition (Shinomol and Muralidhara 2011). Over the past decade, numerous preclinical studies have confirmed these effects, both in the context of pathological and normal, age-related, cognitive decline.

Aqueous extracts of *C. asiatica* seem particularly effective at improving cognitive performance. Kumar et al. (2011) showed that an aqueous extract of *C. asiatica* improved performance in the Morris Water Maze and the elevated plus maze in the mouse D-galactose model of brain aging (Kumar et al. 2011). This improvement was associated with a normalization of acetylcholinesterase activity in the brains of treated animals. Our own lab has also demonstrated that a water extract of *C. asiatica* can improve memory retention in otherwise healthy, aged mice (Gray et al. 2016). In the probe phase of the Morris Water Maze that assesses memory retention, we found that aged, *C. asiatica*-treated male and female mice spent significantly more time in the quadrant that formerly held the platform than did their control, untreated counterparts (Fig. 9). Interestingly, this improvement persisted for 24 h in the females and

72 h in the males after the final day of hidden platform testing. The enhanced memory retention was accompanied by increased expression of the synaptic genes synaptophysin and post-synaptic density protein 95 (PSD95) in the brains of these animals (Gray et al. 2016).

Extracts of *C. asiatica* have also been shown to have beneficial effects for cognitive dysfunction observed in models of Alzheimer's disease (AD). Kumar et al. (2009) demonstrated that a water extract of *C. asiatica* reversed colchicine-induced deficits in elevated plus maze and the Morris Water Maze in rats (Kumar et al. 2009). This is consistent with findings from our own lab where treatment with the water extract of *C. asiatica* attenuated behavioral deficits normally observed in the Tg2576 mouse model of A β -toxicity (Soumyanath et al. 2012). We found that 2 weeks of treatment with the extract normalized time and distance traveled to find the hidden platform in the Morris Water Maze to that of age-matched wild-type animals (Fig. 10).

The neuroprotective effects of *C. asiatica* in the context of beta-amyloid (A β) toxicity have been well documented in vitro. In both PC12 and N2A neuroblastoma cells, as well as in primary rat cortical neurons, extracts of *C. asiatica* prevented A β induced cell death (Chen et al. 2015; Xu et al. 2008). Our lab has demonstrated that an aqueous extract of *C. asiatica* can protect MC65 and SH-SY5Y neuroblastoma cells from A β -induced cytotoxicity (Gray et al. 2014; Soumyanath et al. 2012) and that this protection is accompanied by a decrease in ROS and a normalization of mitochondrial function and calcium homeostasis (Gray et al. 2015). Similarly, in PC12 neuroblastoma cells, this protective effect of an ethanol extract of *C. asiatica* was attributed to inhibition of the production of ROS (Chen et al. 2015), while in N2A cells and cortical neurons the protective effect of the *C. asiatica* extract was thought to be related to enhanced phosphorylation of CREB (cyclic AMP response element binding protein) possibly through ERK (extracellular signal-related kinase) pathway (Xu et al. 2008).

Effects of *C. asiatica* on neuronal health

Impaired arborization and decreased synaptic density are two endpoints widely believed to represent the structural basis of cognitive dysfunction. Therefore,

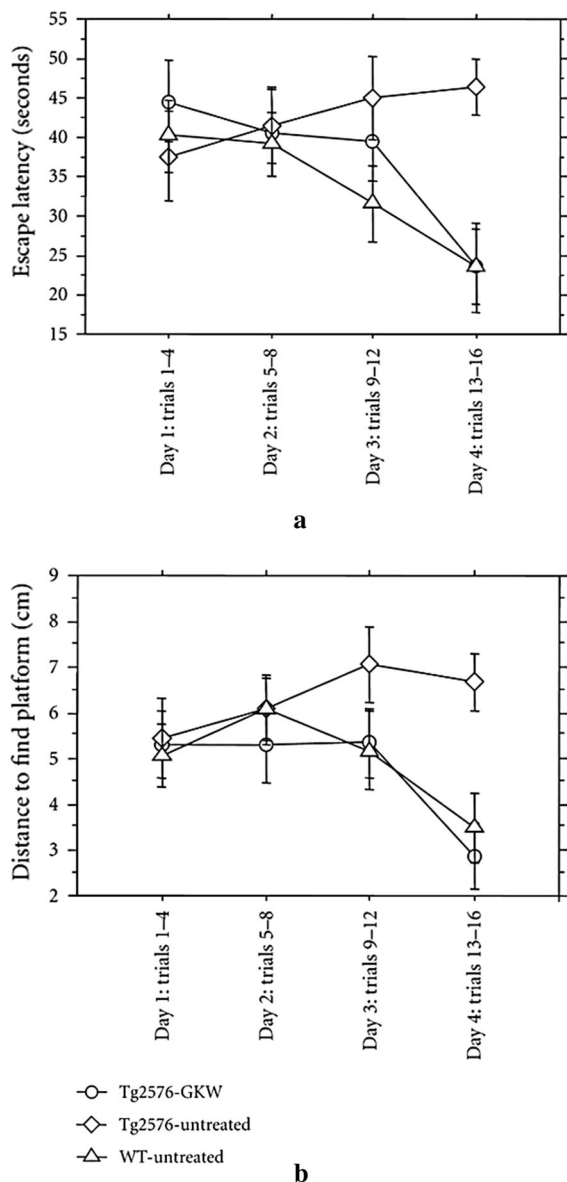


Fig. 10 Morris water maze data of aged female Tg 2576 transgenic or wild type (WT) mice treated with a water extract of *Centella asiatica* (GKW, 2 mg/ml dissolved in the drinking water) for 2 weeks prior to, and during testing. *C. asiatica* treatment normalized memory deficits in the Tg2576 mouse model of A β accumulation, reducing the time and distance traveled by Tg2576 mice to find the hidden platform compared to untreated Tg2576 animals. Adapted from Soumyanath et al. (2012)

the cognitive enhancing effects of *C. asiatica* could result from its effects on neuronal differentiation and dendritic arborization. Extracts of *C. asiatica* have demonstrated neurotogenic properties in various neuroblastoma cell lines (Soumyanath et al. 2005;

Wanakhachornkrai et al. 2013) and Mohandas Rao's group has shown that a fresh leaf extract of *C. asiatica* can enhance dendritic arborization in the hippocampus and amygdala for both neonatal and adult rats (Gadahad et al. 2008; Mohandas Rao et al. 2006, 2009, 2012).

Potential mechanisms of *C. asiatica* associated with neuroprotection and cognitive enhancement

Although the molecular mechanism by which *C. asiatica* exerts its neuroprotective and cognitive enhancing effects is still not fully understood, there are several emerging hypotheses. Normalization of acetylcholinesterase activity has been suggested (Kumar et al. 2011) as have modulations of signal transduction pathways that regulate arborization and synaptogenesis, like ERK1/2 and Akt (Wanakhachornkrai et al. 2013). Another potential candidate mechanism is through inhibition of phospholipase A2 (PLA2). Generation of arachidonic acid by PLA2 is the rate-limiting step in the production of the eicosanoids that play critical roles in the modulation of inflammation and oxidative stress. Increased inflammation, oxidative stress, altered phospholipid metabolism, and increased PLA2 activity are common features of many neurological disorders including Alzheimer's disease, multiple sclerosis, ischemia, epilepsy and spinal cord injury (Farooqui et al. 2006). The water extract of *C. asiatica* has been shown to inhibit multiple PLA2 isoforms in isolated neurons and the brains of treated animals (Barbosa et al. 2008; Defillipo et al. 2012).

In fact, there is growing evidence that antioxidant effects of *C. asiatica*, along with its effects on mitochondrial function, may play an important role in the neuroprotective and cognitive enhancing effects of the plant. This antioxidant activity may contribute to the protection conferred by *C. asiatica* against A β toxicity. In PC12 and IMR32 neuroblastoma cells an ethanol extract of *C. asiatica* reduced A β -induced increases in reactive oxygen species and restored the activity of several antioxidant enzymes that were impaired in response to A β (Chen et al. 2015).

We have demonstrated in SH-SY5Y neuroblastoma cells treated with exogenous A β as well as in MC65 neuroblastoma cells that overexpress amyloid precursor protein that an aqueous extract of *C. asiatica* can reverse the increase in reactive oxygen species

observed in response to A β (Gray et al. 2015). We found that this was accompanied by an increase in the mRNA expression of nuclear factor (erythroid-derived 2)-like 2 (NRF2) (also called NFE2L2) and its target antioxidant response genes. NRF2 is a transcription factor that regulates the endogenous antioxidant response pathway. The endogenous antioxidant response pathway protects cells from oxidative stress (Motohashi and Yamamoto 2004) by increasing transcription of cytoprotective genes (Itoh et al. 1997) through the binding of the transcription factor transcription factor Nrf2 to antioxidant response elements (AREs) in antioxidant gene promoters.

The increased mRNA expression of NRF2 and its target genes suggests that *C. asiatica* can in fact activate the endogenous antioxidant response pathway; however, the exact mechanism by which it does so remains to be elucidated. Further evidence of the antioxidant effects of *C. asiatica* were also observed in vivo following oral administration of an extract of the plant. In the PSAPP mouse model of A β accumulation, treatment with the extract for 8 months resulted in decreased lipid peroxidation and oxidative damage to DNA in the hippocampus of treated animals (Dhanasekaran et al. 2009). Similar in vivo antioxidant effects have been observed in models of healthy aging as well. In the D-galactose model of brain aging, treatment with the aqueous extract of *C. asiatica* decreased lipid peroxidation and restored antioxidant enzyme activity (Kumar et al. 2011). This is consistent with our finding that an aqueous extract of *C. asiatica* increased the expression of NRF2 and its target genes in the brains of healthy, aged mice (Gray et al. 2016).

These antioxidant properties are also thought to contribute to the neuroprotective effects of *C. asiatica* in other neurodegenerative conditions beyond Alzheimer's disease. Huntington's disease, multiple sclerosis, Parkinson's disease and heavy metal exposure share the common features of increased ROS, diminished ATP production and impaired mitochondrial respiration, resulting in synaptic degradation and cognitive impairment (Arun et al. 2016; Conley et al. 2000; Du et al. 2010; Hensley et al. 1994; Navarro and Boveris 2007; Rhein et al. 2009). The antioxidant and mitochondrial effects of *C. asiatica* have been observed in many of these contexts as well. In a rodent model of experimentally induced Parkinsons, treatment with an aqueous extract of *C. asiatica* decreased oxidative damage to lipids and proteins in

the brains of rats treated with the mitochondrial toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and restored levels of antioxidant enzymes (Haleagrahara and Ponnusamy 2010). In a 3-nitropropionic acid (NPA) induced model of Huntington's disease, mice fed *C. asiatica* leaf powder showed lower brain levels of lipid peroxidation, protein carbonyls and ROS, as well as an increase in antioxidant enzyme activity (Shinomol and Muralidhara. 2008b), and decreased oxidative stress in isolated brain mitochondria treated with 3-NPA (Shinomol and Muralidhara. 2008a). *C. asiatica* also protected against brain mitochondrial swelling and partially restored electron transport chain (ETC) enzyme activity in rats (Shinomol and Muralidhara. 2008b).

Similar antioxidant and mitoprotective effects of *C. asiatica* have been observed in response to other neurotoxic insults. *C. asiatica* treatment has been shown to reverse impaired brain mitochondrial enzyme activity, reduce lipid peroxidation and restore antioxidant enzyme activity following exposure to arsenic, aluminum and D-galactose (Flora and Gupta 2007; Kumar et al. 2011; Prakash and Kumar 2013). Additionally, our lab has observed similar mitochondrial effects of an aqueous extract of *C. asiatica* in neuroblastoma cells exposed to A β . We found *C. asiatica* attenuated decreases in ATP production and diminished mitochondrial bioenergetics resulting from A β exposure. We also found that *C. asiatica* increased the expression of genes encoding enzymes in the ETC in cells exposed to A β as well as in healthy cells, suggesting a possible effect on mitochondrial biogenesis (Gray et al. 2015).

Neuroactive effects of constituent compounds from *C. asiatica*

Because extracts of *C. asiatica* are complex mixtures made up of diverse compounds, there is growing interest in identifying compounds within *C. asiatica* that may be responsible for the beneficial effects of entire plant extracts. While it is likely that other compounds will be identified that mediate the positive effects of *C. asiatica*, there is an increasing body of literature suggesting that triterpenes and caffeoylquinic acids (CQAs) found within *C. asiatica* might play an important role in the neuroprotective and cognitive enhancing properties of the plant.

Triterpenes

The triterpenes found within *C. asiatica* have been shown to have similar neuroactive and neuroprotective effects to the entire plant extract in many of the same experimental contexts. For instance, both asiatic acid and asiaticoside have been shown to have anxiolytic properties in rat models (Ceremuga et al. 2015; Wijeweera et al. 2006) without exhibiting sedative effects (Nasir et al. 2012; Wijeweera et al. 2006), as was seen with the *C. asiatica* extracts (Chanana and Kumar 2016; Wanasuntronwong et al. 2012). Asiatic acid, asiaticoside and madecassoside also have neuroprotective effects in models of stroke showing anti-inflammatory effects including reduced cytokine levels and microglial activation (Chen et al. 2014; Krishnamurthy et al. 2009; Luo et al. 2014). Both madecassoside and asiatic acid also reduced infarct volume and attenuated the neurological deficits associated with the ischemia (Krishnamurthy et al. 2009; Luo et al. 2014).

Asiatic acid and asiaticoside have also been shown to have neuroprotective properties against neurotoxic insults. In rat primary cortical neurons, asiatic acid protected the cells against C2 ceramide-induced apoptosis while asiaticoside was protective against ischemia-induced cell death (Sun et al. 2015; Zhang et al. 2012a). There is conflicting literature about whether these compounds can protect against A β toxicity. There are reports that both asiatic acid and asiaticoside are protective against A β -induced cytotoxicity in glial cells (Jew et al. 2000; Mook-Jung et al. 1999) yet in our lab none of the triterpenes found in *C. asiatica* showed any protective effect in MC65 cells against A β -induced cytotoxicity (Gray et al. 2014)

Asiatic acid, asiaticoside and madecassoside also exert cognitive enhancing properties in various models of cognitive impairment. Asiaticoside improved learning and memory deficits caused by ischemia reperfusion (Chen et al. 2014), as well as those related to aging in the senescence-accelerated prone mouse strain (SAMP8) mouse model of accelerated senescence (Lin et al. 2013). In both cases, the beneficial effects of asiaticoside were thought to be related to effects of kinase signaling pathways, decreasing p38MAPK activation in the stroke model, and increasing PKA and calmodulin-dependent protein kinase (CAMK) activation in the aged mice. Madecassoside also inhibited the p38MAPK signaling

pathway and attenuated cognitive impairment in the D-galactose model of brain aging (Lin et al. 2014). Both asiaticoside and madecassoside have been shown to increase expression of proteins related to synaptic plasticity (Lin et al. 2013, 2014).

Asiatic acid improved both valproic acid (VPA)- and glutamate-induced cognitive impairment in mice (Umka Welbat et al. 2016; Xu et al. 2012). In the VPA-treated animals this effect was attributed to reduced hippocampal histone deacetylase activity, while in the glutamate-treated mice the cognitive improvement was thought to be related to antioxidant activities of asiatic acid. Asiatic acid has also been shown to enhance cognitive performance in healthy rodents, improving learning and memory and even increasing cell proliferation in the hippocampus of adult rats (Nasir et al. 2011; Sirichoat et al. 2015). The increased proliferation was accompanied by increased Notch1 protein levels (Sirichoat et al. 2015). Asiatic acid has also been shown to be neuroprotective in the context of spinal cord injury where treatment resulted in increased expression of Nrf2 and its target genes along with decreased levels of reactive oxygen species and a more robust physical recovery (Jiang et al. 2016).

Caffeoylquinic acids (CQAs)

CQAs also possess many of the same neuroprotective properties that are seen with *C. asiatica* extracts against a variety of toxic insults. The mono-CQA chlorogenic acid has been shown to protect primary mouse cortical neurons from glutamate excitotoxicity, possibly through its ability to normalize calcium homeostasis in these cells (Mikami and Yamazawa 2015). The diCQAs 3,4-diCQA, 3,5-diCQA and 3,5-diCQA methyl ester were similarly able to prevent glutamate-induced increases in calcium, reactive oxygen species and protect against cell death in rat cortical neurons (Kim et al. 2012a). The CQA derivative 1,5-O-dicaffeoyl-3-O-[4-malic acid methyl ester]-quinic acid has also been shown to be neuroprotective in SHSY5Y neuroblastoma cells treated with NMDA. This compound inhibited NMDA-induced phosphorylation of ERK1/2, p38 MAPK and JNK1/2 m while also restoring the activation of CREB, AKT and GSK3B that had been impaired by the treatment (Tian et al. 2015).

Our lab has shown that diCQAs can protect against A β -induced cytotoxicity. Both 1,5-diCQA and 3,5-diCQA potently protected both MC65 and SH-SY5Y neuroblastoma cells from A β -induced cell death and induced the expression of NRF2 and its target genes. 3,5-diCQA also enhanced both basal and maximal mitochondrial respiration and significantly increased the expression of genes encoding enzymes in the electron transport chain suggesting a possible effect on mitochondrial biogenesis (Gray et al. 2015).

CQAs and their metabolites can also exert cognitive enhancing effects in many of the same model systems where treatment with *C. asiatica* was beneficial. Caffeic acid, for instance, reduced infarct volume, attenuated neurological deficits and improved working, spatial and long-term aversive memory deficits in a mouse model of stroke (Pinheiro Fernandes et al. 2014). The same compound was found to be protective in the context of global cerebral ischemia–reperfusion injury in rats where caffeic acid treated animals had diminished memory deficits, decreased pro-inflammatory markers and lipid peroxidation, increased antioxidant enzyme activity and reduced hippocampal cell injury and death. In this model, caffeic acid also reduced 5-lipoxygenase expression leading to the hypothesis that the neuroprotective effects may have resulted from the interaction with that enzyme, which metabolizes arachidonic acid into the pro-inflammatory leukotrienes (Liang et al. 2015).

CQAs have also been shown to improve age-related cognitive impairment. In the SAMP8 mouse model of aging both a CQA-rich purple sweet potato extract and the individual compound 3,5-diCQA improved learning and memory deficits. In both cases this was accompanied by an increased expression of glucose metabolism enzymes (Han et al. 2010; Sasaki et al. 2013). The cognitive enhancing properties of CQAs have been observed in many rodent models of Alzheimer’s disease as well. Treatment with caffeic acid or a diet rich in caffeic acid improved memory in the Tg2576 mouse model of A β accumulation as well as in A β _{25–35} injected mice and A β _{1–40} injected rats. These cognitive improvements were associated with increased synaptophysin expression and reduced markers of inflammation, oxidative damage and p38MAPK activity (Kim et al. 2012a; Subash et al. 2015; Wang et al. 2016). Caffeic acid also restored streptozotocin-induced memory deficits in rats and acetylcholinesterase activity (Deshmukh et al. 2016).

The mono-CQAs chlorogenic acid and 3-O-caffeoylquinic acid improved memory in scopolamine and A β _{1–42} treated mice respectively. Both compounds decreased lipid peroxidation in the brains of treated animals and chlorogenic acid treatment also inhibited acetylcholinesterase activity in the hippocampus and cortex of the animals (Choi et al. 2014; Kwon et al. 2010).

Neurological effects of *Centella asiatica* in humans

Cognition

Centella asiatica has been used in traditional systems of medicine for hundreds of years to treat a multiplicity of conditions including infectious diseases, insomnia, hypertension, asthma, infertility and dementia (Brinkhaus et al. 2000). Practitioners of traditional medicine use local *C. asiatica* plants prepared in a variety of ways, introducing processing-induced variation in the active constituents to the genetic and geographical factors noted previously (“[Chemical variation in *Centella asiatica*](#)” section). These variations make it challenging to derive direct correlations between scientific studies and traditional usage.

While there is historical usage and extensive preclinical literature on *C. asiatica*’s neurological properties, rigorous human trials are lacking. Our literature search revealed fewer than ten human studies focusing on neurological disorders (Table 4a and 4b). Each of these studies reported improvements in cognition (Appa Rao et al. 1973; Dev et al. 2009; Farhana et al. 2016; Rakesh et al. 2013; Tiwari et al. 2008; Wattanathorn et al. 2008), mood (Bradwejn et al. 2000; Jana et al. 2010; Wattanathorn et al. 2008) and/or quality of life (Mato et al. 2011; Tiwari et al. 2008) with *C. asiatica* supplementation. One of the studies noted differences in cognitive outcomes based upon gender where the males had improvements in three out of eight cognitive measures (long-term retrieval, visual spatial thinking and speed processing), whereas females had improvements in five measures (long-term retrieval, short-term memory, working memory, executive process and delayed recall) (Dev et al. 2009). In two separate clinical trials included in Table 4b, the subject population was primarily female. Given the observed gender differences by Dev et al. (2009), incorporation of adequate

Table 4a Study location and products used for human trials of *Centella asiatica* assessing cognition, mood and quality of life

Trial Number	Study Location	CA Material Given	CA Source Country	Standardization	References
1	India	Tablets of shade-dried powdered whole plant	India	Not provided	Appa Rao et al. (1973)
2	Canada	Crude stem and leaf powder by Nature's Way	Madagascar (personal communication with Nature's Way)	None (personal communication with Nature's Way)	Bradwejn et al. (2000)
3	Thailand	Capsules of "specialized" aerial part extract	Thailand	Total phenolic content (tannic acid equivalents) = 29.9 mg/g; asiaticoside 1.09 mg/g and asiatic acid 28.89 mg/g of crude extract	Wattanathorn et al. (2008)
4	India	Capsules of dried and ground leaves	India	Not provided	Tiwari et al. (2008)
5	Malaysia	Capsules of crude herb	Not provided	Not provided	Dev et al. (2009)
6	India	Capsules of 70% hydro-ethanolic extract of dried aerial parts	India	Ursolic acid 1.5–1.89% of plant extract	Jana et al. (2010)
7	Thailand	Capsules of a blended aqueous leaf extract combined with sugar (Patent 4721; Thailand)	Thailand	Total phenolic content (tannic acid equivalents) = 29.9 mg/g; asiaticoside 1.09 mg/g and asiatic acid 48.89 mg/g of crude extract	Mato et al. (2011)
8	India	Capsules of "Ghava Satva"	Not provided	Not provided	Rakesh et al. (2013)
9	Indonesia	Capsules of dried 70% hydro-ethanolic whole herb extraction	Not provided	"Standardized dry form"	Farhana et al. (2016)

CA, *Centella asiatica*

Table 4b Results of human trials using *Centella asiatica* to assess changes in cognition, mood and quality of life*

Trial number	Product dose/duration	Study design	Control	Subjects	Assessment	Authors' conclusions
1	500 mg daily/3 months	Double-blind; randomized	Starch placebo	30 mentally-retarded children (age 7–18 years; both genders)	Binet-Kamat Test, adapted Weschler intelligence scale for children	▲ IQ, ▲ behavior
2	12 g dissolved in grape juice/once	Double-blind; randomized	Grape juice placebo	40 healthy adults (age 18–45; 21 males, 19 females)	Acoustic startle response, 100-mm visual analog scale for moods, heart rate and blood pressure	▼ Startle response, ▲ self-rated energy level
3	250, 500 or 750 mg daily/once and for 2 months	Double-blind; randomized	Placebo	28 healthy elderly (mean age 65.05 ± 43.56 years; 4 males, 255 females)	Modified CDR test battery; 16 visual analogue scales of Bond-Lader	▼ Reaction time, ▲ accuracy of working memory, ▲ alertness and calmness
4	500 mg twice daily/6 months	Open-label	None	60 elderly with mild cognitive impairment (age >65 years; genders not reported)	MMSE, ADL scale, IADL, caregiver interviews, Yesavage GDS	▲ MMSE score, ▼ diastolic BP, ▲ appetite and sense of well-being, ▲ sleep, ▼ anxiety, ▼ peripheral neuritis
5	500 mg/10 kg body weight daily/2 months	Randomized; blinding not discussed	Corn starch placebo	41 healthy middle-aged adults (age 35–50 years; 19 males; 22 females)	Woodcock-Johnson Cognitive Abilities Test III	▲ Cognitive ability scores (3/8 tests in males; 5/8 in females)
6	500 mg twice daily after meals/2 months	Open-label	None	33 adults with generalized anxiety disorder (mean age 33 years; 18 males, 15 females)	SF-36 health survey questionnaire, validated stress scale, anxiety scale, depression scale, adjustment scale and attention scale	▼ Anxiety, ▲ self-perceived stress, ▼ depression index, ▲ adjustment index, ▲ attention level
7	250, 500, or 750 mg daily/90 days	Double-blind; randomized	Placebo	80 healthy elderly (mean age 65.05 ± 3.56 years; 4 males, 76 females)	30 second chair stand test, hand grip, six minute walk test, SF-36 quality of life questionnaire	▲ Lower extremity strength and life satisfaction subscale with 500 and 750 mg doses
8	500 mg twice daily after meals with warm water/6 months	Open-label	None	25 elderly with MMSE < 25 (age > 60 years; gender not reported)	MMSE, caregiver interviews, vitals	▲ MMSE score, ▼ mean systolic and diastolic blood pressure
9	1000 or 750 mg daily/6 weeks	Double-blind; non-randomized	3 mg folic acid	48 subjects with post-stroke vascular cognitive impairment (mean age 60.27 ± 11.83 years; 60.41% males, 39.58% females)	MoCA-Ina	▲ Delayed recall memory

*Trial numbers, product used, and references correspond to those in Table 4a

MoCA-Ina, Montreal Cognitive Assessment-Indonesian version; MMSE, Mini Mental State Examination; CDR, Clinical Dementia Rating; ADL, Activities of Daily Living; GDS, Geriatric Depression Scale; IADL, Instrumental Activities of Daily Living; ▲, increased or improved; ▼ decreased

subjects from both genders is warranted in future studies.

Unfortunately, the studies reviewed used products with a variety of preparation methods and some lack an adequate description of the product used, and/or incorporate no means of standardization (Table 4a), thereby limiting the ability to compare between studies. In addition, the studies were of variable quality and relevance. Some did not include a placebo or control group, and some assessed cognitive changes in a cognitively healthy elderly or middle-aged subject population, limiting clinical applicability to states of cognitive impairment (Table 4b). Despite these issues, the overall positive effect of the herb in these studies is encouraging. The absence of reports of adverse effects in any of these human studies is also reassuring, and corroborates the classification of *C. asiatica* as a Class 1 herb (one that can safely be consumed when used appropriately) in the Botanical Safety Handbook (McGuffin et al. 1997).

Clearly the extensive preclinical data and the positive results seen in the limited human studies so far warrants more rigorous human trials. Ideally, these would investigate the effects of authenticated and chemically well-characterized *C. asiatica* products, that have been standardized to known active compounds and are administered at doses based on interspecies scaling from preclinical models. Clinical studies would optimally include a control group, adequate subjects from both genders and the appropriate study population for the outcome of interest.

Microcirculation

A separate series of human trials of *C. asiatica* have focused on its dermatologic and vascular applications (Brinkhaus et al. 2000). Several placebo-controlled, randomized clinical studies demonstrated the beneficial effects of *C. asiatica* on peripheral microcirculation using a preparation known as TTFCA. TTFCA contains 40% asiaticoside and 60% combined asiatic acid and madecassic acid (Grimaldi et al. 1990). Doses of 60–180 mg of TTFCA daily for 4–24 weeks improved microcirculation and decreased associated symptoms such as edema in hypertensive microangiopathy (Cesarone et al. 2001; Incandela et al. 2001). In addition, 60 mg of TTFCA twice daily for six months increased capillary permeability and microcirculatory parameters such as resting skin flux,

venoarteriolar response, transcutaneous oxygen and carbon dioxide and rate of ankle swelling) in diabetic patients (Belcaro et al. 2011; Cesarone et al. 2001). Further studies have found that TTFCA protects the venous endothelium from oxidative damage (Cesarone et al. 2001).

While these studies examined *Centella's* triterpenes' effects on the peripheral system via endothelial restoration and anti-oxidation, the brain also possesses a complex microvasculature communicating primarily via endothelial tight junctions (Drewes 2001). To date, there have been no studies on the effects of TTFCA or other *C. asiatica* products on brain perfusion; however, there have been reports of brain bioavailability of the triterpenes, asiatic acid and madecassic acid in mouse models (Yin et al. 2012). Based upon this, it can be hypothesized that *C. asiatica* may affect the microvasculature within the brain, but this aspect requires further investigation.

Absorption, distribution, metabolism and elimination (ADME) of active compounds of *C. asiatica*

The complex composition of botanical materials renders ADME studies of their multiple biologically active compounds in humans or animal models particularly challenging. To date, in vivo ADME studies of *C. asiatica* have primarily focused on the *Centella*-specific triterpenoids asiaticoside, madecassoside and their aglycones asiatic acid and madecassic acid. No reports were found of studies examining the ADME of caffeoylquinic acids (CQAs) or flavonoids after administration of *C. asiatica*; however, there is literature on the ADME of these latter groups of compounds derived from other botanical sources.

Triterpenes

Using sensitive methodologies, such as high-performance liquid chromatography (HPLC) coupled to ultraviolet detection (LC-UV) or tandem mass spectrometry (LC-MSⁿ), many have been able to detect and quantify triterpenoids and their metabolites within human and animal biological matrices (Han et al. 2012; Leng et al. 2013; Sharma et al. 2011; Thongnopnua 2008; Wang et al. 2014; Xia et al. 2015). In humans, bioavailability studies of *C. asiatica*

have focused on asiaticoside and asiatic acid. Pharmacokinetic studies have reported that the time to reach the maximum plasma concentration (t_{\max}) for asiatic acid was 4.0–4.6 h post-ingestion of either purified asiatic acid or the triterpene mixture, TTFCA (Grimaldi et al. 1990; Rush et al. 1993). Rush et al. (1993) found that the separate administration of equimolar oral doses of asiatic acid (12 mg) and asiaticoside (24 mg) in humans resulted in a similar total concentration (area under the curve) of plasma asiatic acid over 12 h. Of note, the peak plasma concentration was attained earlier when asiatic acid was administered compared to asiaticoside. The asiatic acid plasma profile also demonstrated more of a typical “sawtooth pattern” compared to asiaticoside suggestive of more immediate availability, compared to the delay required for in vivo hydrolysis of asiaticoside by intestinal enzymes (Rush et al. 1993). This is also seen in a separate human study by Grimaldi et al. (1990), where there was no observed difference in the AUC of asiatic acid after the administration of pure asiatic acid and asiaticoside independently. In this study, the investigators also compared a single administration to a 7-day course of treatment, in which they found the area under the curve over 24 h was much greater for chronic treatment than single treatment despite the short half-life. They propose this is due to slow metabolism of asiaticoside from previous doses, either because of delayed absorption or delayed metabolism.

Since most *C. asiatica* preparations are not composed of purified compounds, further studies are needed to understand the pharmacokinetics of triterpenes when present in complex extracts. One study has been conducted in a dog model using an encapsulated water extract of *C. asiatica* standardized to the amount of asiaticoside. In this study, the peak concentration of asiatic acid was observed at 2.7 h after consumption (Zheng and Wang 2009). As with the human study, this model demonstrated little asiaticoside within the plasma after oral administration due to a proposed complete biotransformation into asiatic acid and its glucuronide and sulfate conjugates (Rush et al. 1993; Zheng and Wang 2009).

Studies in rats have shown the absolute oral bioavailability of *C. asiatica*'s triterpenoids is 16–30% for the glycosides and 50% for the acids (Chassaud et al. 1971; Vogel et al. 1990; Yuan et al. 2015) with primary elimination via the feces (Leng

et al. 2013). These low levels are proposed to be due to poor solubility, slow absorption, dissolution within the gastrointestinal tract, and/or variations in gastrointestinal transit time (Chassaud et al. 1971; Yuan et al. 2015). Bioavailability studies in rats using pure triterpenoid compounds, or ethanolic extracts of *C. asiatica* aerial parts, have revealed similarities in the observed pharmacokinetic profile of madecassoside (Han et al. 2012; Leng et al. 2013; Wang et al. 2014), madecassic acid (Wang et al. 2014), and asiatic acid (Nair et al. 2012; Yuan et al. 2015). Most of the studies reported a rapid time to maximum concentration ($T_{\max} = 0.5\text{--}1.3$ h) and a delayed second maximum concentration peak in plasma levels and/or tissue distribution due to a proposed enterohepatic recirculation and/or selective and differential absorption from the gastrointestinal tract (Han et al. 2012; Leng et al. 2013; Nair et al. 2012; Wang et al. 2014). Notably, unlike the human studies on asiaticoside described earlier (Grimaldi et al. 1990; Rush et al. 1993), the glycoside madecassoside was reported to be present in the plasma of rats following oral administration (Han et al. 2012; Leng et al. 2013; Wang et al. 2014). Of these studies, Wang et al. (2014) also found the amount detected in plasma was affected by the disease state. In their rat model of arthritis, they noted higher levels of madecassic acid and lower levels of madecassoside in rats with induced arthritis, compared to non-arthritic rats (Wang et al. 2014). They propose this may be due to a wider distribution of the madecassoside and decreased elimination in vivo due to the disease.

Xia et al. (2015) used the well-established zebrafish model and LC/IT (ion trap)-MSⁿ to identify any downstream metabolites from the aglycones. Using this sensitive methodology, they detected ten different phase one metabolites from asiatic acid and nine from madecassic acid, derived from hydroxylation and/or dehydrogenation reactions in vivo (Xia et al. 2015). It is hypothesized that these metabolites may be more bioactive compared to their parent compounds. The occurrence and biological activity of these metabolites in human and other mammalian systems requires further study.

Application to neurological diseases

Few studies have looked at the tissue distribution of the triterpenes following oral administration, in

particular brain levels of these compounds. One study detected asiatic acid and madecassic acid within the brains of mice (Yin et al. 2012). Another focused only on madecassoside levels, which were reported to be below the lower limit of quantification in the brains of rats (Leng et al. 2013). Given the previously discussed observed cognitive changes and brain biochemical effects seen in animal models with *C. asiatica* administration, further investigation is needed to understand the brain bioavailability of these compounds and their metabolites.

Caffeoylquinic acids (CQAs)

There have been no reported studies on the ADME of CQAs following oral administration of *C. asiatica*, but there are multiple studies on the fate of these compounds when derived from other botanical sources (Williamson et al. 2011). Coffee, in particular, is a major source of CQAs, being rich in 3-, 4- and 5-CQA as well as the 3,4-, 3,5- and 4,5-diCQA isomers. Controversial data exists on the oral absorption of the CQAs (Williamson et al. 2011). In humans, high plasma levels of intact mono and diCQAs were reported following coffee consumption (Farah et al. 2008; Monteiro et al. 2007) (Farah et al. 2008). Both 1,5-diCQA and its methylated metabolite, 1,5-diferuloylquinic acid, were measured in human plasma following oral administration of 1,5-diCQA (Liu et al. 2010). DiCQAs were also found in rat plasma after oral administration of *Ainsliaea fragrans* extract containing 1,5-, 3,4-, 3,5- and 4,5-diCQA (Su et al. 2014). By contrast, intact CQAs were not seen in the plasma after oral administration of artichoke extracts known to contain both mono and di-CQAs (Wittemer et al. 2005). It has been suggested that absorption is dose dependent; at higher doses, a proportion of the CQAs may escape metabolism or hydrolysis due to enzyme saturation (Erk et al. 2012) facilitating their appearance in the plasma. It has also been noted, that mono and di-CQAs can isomerize in vivo (Basavara-jappa et al. 2014; Kahle et al. 2007). A comparison of bioavailability and pharmacokinetic data for CQAs and metabolites from several studies (Williamson et al. 2011) identifies several other discrepancies and highlights the need for dose response studies.

The metabolism of CQAs is well documented (Erk et al. 2012; Stalmach et al. 2009; Williamson et al. 2011; Wong et al. 2010) and includes hydrolytic

cleavage of caffeic acid moieties from the quinic acid group, Phase I methylation of the caffeic acid to ferulic and isoferulic acid, reduction of the double bond in hydroxycinnamic acids by colonic microflora to produce dihydrocaffeic, dihydroferulic and dihydroisoferulic acids, Phase II sulfation or glucuronidation of the hydroxyl groups of any these compounds, and glycine conjugation with the free carboxylic acid group of the hydroxycinnamic acids. Lactone forms of mono CQAs have also been identified (Stalmach et al. 2009). Further breakdown of caffeic acid to ethyl, methyl and vinyl catechols by gut microflora has been proposed (Senger et al. 2016). Urinary excretion products include the CQA metabolites rather than the CQAs themselves, mostly as sulfate and glucuronide conjugates (Stalmach et al. 2009). Total urinary metabolites corresponded to approximately 30% of the CQAs intake from coffee (Stalmach et al. 2009).

A LC-MSⁿ method capable of quantifying 56 compounds that may be derived from CQA metabolism in human plasma has been reported (Marmet et al. 2014). Using this method, Scherbl et al. (Scherbl et al. 2017) reported the pharmacokinetics in humans of mono CQAs and various metabolite groups listed earlier following consumption of coffee. Considerable inter-subject variation was noted in pharmacokinetic parameters. Peak plasma levels for the mono CQAs and hydroxycinnamic acid metabolites were generally rapidly attained ($t_{\max} \leq 1$ h) suggesting their absorption from the upper gastrointestinal tract, whereas the dihydro metabolites of the hydroxycinnamates peaked significantly later (5.5–8.5 h), supporting their later formation in the colon. Similar results were found in other studies on CQAs from coffee (Stalmach et al. 2009) and artichoke extract (Wittemer and Veit 2003). Peak urinary excretion of CQA metabolites was reported in the 6–12 h (Scherbl et al. 2016) or 5–8 h (Stalmach et al. 2009) sample collections.

A pharmacokinetic and tissue distribution study of mono and diCQAs from *Ainsliaea fragrans* in rats (Su et al. 2014) reported mono CQAs were undetectable in the plasma, but that diCQA levels peaked once at less than 30 min and again at around 4 h, possibly due to enterohepatic circulation. Mono and diCQAs and caffeic acid were detected in multiple organs (lung, liver, muscle, brain, spleen, kidney and heart). Peak brain levels of individual diCQAs ranged from 0.2 to 20 $\mu\text{g/g}$ following oral administration of the *A. fragrans* extract (46 mg/kg b.wt total diCQAs). Based

on this data, the *C. asiatica* oral doses (2.5 mg/kg b. wt. total diCQAs) used in our in vivo studies (Soumyanath et al. 2012) (Gray et al. 2016) could potentially give brain levels of up to 1000 ng/g of individual diCQAs. This is comparable to the minimum neuroprotective concentration (500 ng/ml) we have observed in vitro for these compounds (Gray et al. 2014). Caffeic acid and ferulic acids levels in the brain were significantly increased after injection of an intravenous bolus of these compounds to rats (Gasperotti et al. 2015). Ferulic acid but not caffeic acid was also found in rat brains after oral administration of *Portulaca oleracea* extract, which contains both compounds (Zhang et al. 2015). The detection of these CQAs and metabolites in the brain supports their potential to penetrate the blood brain barrier after administration of *C. asiatica*, and suggests that in vitro effects seen for some of these compounds on neuronal cultures may be recapitulated in vivo.

Flavonoids

The flavanols catechin, epicatechin, the flavone rutin, and flavonols quercetin and kaempferol have been detected within extracts of *C. asiatica* (Alqahtani et al. 2015; Chandrika and Prasad Kumarab 2015; Lu et al. 2011). To our knowledge, there have not been any studies on the ADME of these flavonoids derived specifically from *C. asiatica*; however, there have been multiple studies of flavonoids derived from other plant materials, such as food sources (Hollman 2004; Kumar and Pandey 2013).

With the exception of the catechins, most flavonoids are bound to sugars within plant material making them β -glycosides (Kumar and Pandey 2013). These glycosides are primarily absorbed after being deglycosylated in the small intestinal lumen and by colonic microflora (Lu et al. 2013; Németh et al. 2003; Yang et al. 2012), while the catechins, aglycones and hydrophilic compounds are absorbed via the small intestine mucosa (Hollman et al. 1999). Following absorption, the compounds are conjugated in the liver to form *O*-methylated, *O*-sulfated and *O*-glucuronidated conjugates, which have been detected in the plasma of rats and humans (Actis-Goretta et al. 2012; Bravo 1998; Hollman et al. 1999; Hollman 2004; Jäger and Saaby 2011).

Rats have been used to assess the brain distribution of these compounds. Quercetin and kaempferol

aglycones and conjugates were found in the brain following the consumption of the botanical extract *Gingko biloba* (Rangel-Ordóñez et al. 2010). Similar studies are needed to assess the brain bioavailability of flavonoids from *C. asiatica* and to further understand the role these compounds play in its effect on cognition and mood.

Conclusions and perspective

Centella asiatica demonstrates remarkable cognitive, neurotropic and neuroprotective effects. These properties support the traditional use of the herb as a memory enhancer, and highlight its potential to modulate disease processes involved in neurodegenerative disorders. Successful development of novel treatments based on *C. asiatica* will require a focus on at least three main areas of research. First, a more extensive understanding of the active compounds of *C. asiatica* is required. To date, the triterpenes asiatic acid, asiaticoside, and madecassoside, as well as caffeoylquinic acids, have been shown to contribute to the neurological effects of the herb. At the present time, these two groups of compounds are the most relevant for standardization of *C. asiatica* products. However, further research, using bioassay guided fractionation approaches, or OPLS-DA activity/fingerprint correlations may identify additional active compounds within *C. asiatica*. Interactions between the different compounds (i.e. synergy, additivity, antagonism) also need to be explored. Second, the detailed biochemical mechanisms underlying the observed effects on mitochondrial activity, oxidative stress, neurotransmitter homeostasis, and synaptic plasticity need to be defined as does the manner in which each of these effects contributes to the broader neuroprotective and cognitive enhancing properties of the plant. This will be a challenge when dealing with complex extracts where the multiple components could simultaneously influence multiple pathways. A careful evaluation of individual active compounds and known mixtures to evaluate interactions will be required. Finally, human studies of *C. asiatica* extracts, or compounds derived from the botanical, will be important to confirm the clinical significance of earlier studies. Adequate chemical characterization and description of the *C. asiatica* test material will be vital for these studies, both to ensure the presence of

adequate levels of active compounds, and to allow comparison between studies. This is particularly important given the noted variation in content of phytochemicals in *C. asiatica* due to genetic and geographical factors and processing methods. The use of contemporary analytical techniques such as LC-HRMS and IM-HRMS will be particularly important for the fingerprinting of extracts and documentation of known and unknown compounds.

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