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Employing Two-stage Derivatisation and GC–MS to Assay for Cathine and Related Stimulant Alkaloids across the Celastraceae

Luke R. Tembrock,[a]* Corey D. Broeckling,[b] Adam L. Heuberger,[c] Mark P. Simmons,[a] Frank R. Stermitz[d] and Jessica M. Uvarov[a]

ABSTRACT:

Introduction – *Catha edulis* (qat, khat, mirra) is a woody plant species that is grown and consumed in East Africa and Yemen for its stimulant alkaloids cathinone, cathine and norephedrine. Two Celastraceae species, in addition to qat, have been noted for their stimulant properties in ethnobotanical literature. Recent phylogenetic reconstructions place four genera in a clade sister to *Catha edulis*, and these genera are primary candidates to search for cathine and related alkaloids.

Objective – Determine if cathine or related alkaloids are present in species of Celastraceae other than *Catha edulis*.

Methods – Leaf samples from 43 Celastraceae species were extracted in water followed by basification of the aqueous extract and partitioning with methyl-*t*-butyl ether to provide an alkaloid-enriched fraction. The extract was derivatised in a two-stage process and analysed using GC–MS for the presence of cathine. Related alkaloids and other metabolites in this alkaloid-enriched fraction were tentatively identified.

Results – Cathinone, cathine and norephedrine were not detected in any of the 43 Celastraceae species assayed other than *Catha edulis*. However, the phenylalanine- or tyrosine-derived alkaloid phenylethylamine was identified in five species. Nine species were found to be enriched for numerous sterol- and terpene-like compounds.

Conclusion – These results indicate that cathine is unique to *Catha edulis*, and not the compound responsible for the stimulant properties reported in related Celastraceae species.

Keywords: *Catha edulis*; GC–MS; khat; qat; stimulant phenethylamine alkaloids

Introduction

The plant family Celastraceae contains bioactive metabolites including insecticides, anti-cancer agents, antibacterial compounds, anti-diabetic drugs and central nervous system stimulants (e.g. Brünning and Wagner, 1978; Yoshikawa *et al*., 1997; Spivey *et al*., 2002; López *et al*., 2011). *Catha edulis* is the only Celastraceae species that is cultivated specifically for its stimulant alkaloids. Qat, as it is commonly known and correctly transliterated from Arabic (Varisco, 2004), is mainly cultivated and/or collected from the wild and consumed in Ethiopia, Israel, Kenya, Somalia, Tanzania, Uganda and Yemen. Qat is generally consumed by chewing the fresh young leaves and shoots followed by ingestion of the liquid constituents. The young shoot tips of qat contain the stimulant alkaloids (*S*)-cathinone (**1**), hereafter cathinone (United Nations Narcotics Laboratory, 1975; Schorno and Steinegger, 1979; Szendrei, 1980), (1*R*,2*S*)-norephedrine (**2**) (United Nations Narcotics Laboratory, 1975), hereafter norephedrine, and (1*S*,2*S*)-norpseudoephedrine (**3**), hereafter cathine (Wolfes, 1930) (Fig. 1).

The structural similarity of cathinone and cathine to amphetamine (**4**; Fig. 1) results in similar actions to the human central nervous system as amphetamine (Patel, 2000; Hoffman and Al'Absi, 2010). As shoots develop, cathinone is converted enzymatically into cathine, which is found in young leaves and stems (Krizevski *et al*., 2007). A similar pattern of phytochemical biosynthesis correlated with developmental stage of plant parts has been found in *Ephedra sinica* (Ma Huang; Krizevski *et al*., 2010). Due to the rapid conversion of cathinone into the less potent cathine after harvest, qat is treated as a perishable commodity and is usually consumed within two or three days after harvest, with the highest value being given to the freshest leaves (Altabachew *et al*., 2013).

The only other species from which cathinone and cathine have been detected are *Ephedra gerardiana* and *Ephedra sinica* (Ephedraceae) (Grue-Sørensen and Spenser, 1994; Krizevski *et al*., 2010). The presence of cathinone and cathine in *Ephedra* is a clear demonstration of convergent evolution given that *Ephedra* is a gymnosperm and qat an angiosperm (e.g. Stevens, 2012). In *Ephedra* the pathway has further steps in which cathine and norephedrine are *N*-methylated, yielding pseudoephedrine (**5**) and ephedrine (**6**) (Fig. 1; Krizevski *et al*., 2010). The presence of cathine in relatively high levels in qat is likely due to the lack of *N*-methylase enzymes, resulting in the deposition of cathine in older qat tissues instead of ephedrine alkaloids, as in *Ephedra*

* Correspondence to: Luke R. Tembrock, Department of Biology, Colorado State University, Fort Collins, CO 80523, USA. Email: 101020tembrock@gmail.com

[a] Department of Biology, Colorado State University, Fort Collins, CO, 80523, USA

[b] Proteomics and Metabolomics Facility, Colorado State University, Fort Collins, CO, 80523, USA

[c] Department of Horticulture and Landscape Architecture, Colorado State University, Fort Collins, CO, 80523, USA

[d] Department of Chemistry, Colorado State University, Fort Collins, CO, 80523, USA

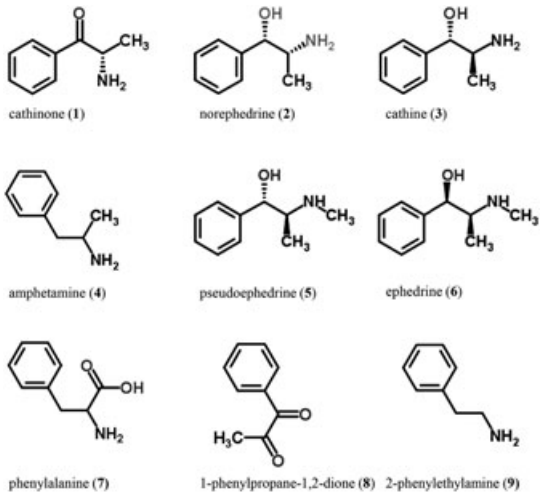

Figure 1. Molecular structures of cathinone (**1**), norephedrine (**2**), cathine (**3**), amphetamine (**4**), pseudoephedrine (**5**), ephedrine (**6**), phenylalanine (**7**), 1-phenylpropane-1,2-dione (**8**) and 2-phenylethylamine (**9**).

(Krizevski *et al*., 2007). Although cathinone and cathine are currently only known from a single angiosperm species, similar phenethylamine alkaloids produced from comparable biosynthetic pathways have been found in approximately 40 vascular-plant families and to a lesser degree in some fungal and algal lineages (Smith, 1977; Kuklin and Conger, 1995; Kulma and Szopa, 2007). Enzymes able to convert amino acids like phenylalanine (**7**) into alkaloid precursors and alkaloids have been found to evolve after duplication of genes coding for primary metabolic enzymes (Nakajima *et al*., 1993; Ober and Hartmann, 1999). After these secondary metabolic pathways evolved, diversification of end-product alkaloids is thought to result from minor changes to enzymes in the pathway (Nakajima *et al*., 1993). This diversification of alkaloid structures can be species- or even ecotype-specific (Ziegler *et al*., 2009). As such, the presence or absence of different types of alkaloids have been useful characters for chemosystematic analyses (e.g. Gomes and Gottlieb, 1980).

Despite ongoing phytochemical research in Celastraceae, a systematic investigation to identify cathinone and cathine as well as precursors and possible derivatives thereof has not been undertaken in this family. Much of the phytochemical research in Celastraceae has focussed on molecules, such as terpenoids, that have unique structures and potential as pharmaceutical products (reviewed in Shan *et al*., 2013). Here, the presence of cathine in Celastraceae species other than *Catha edulis* is hypothesised based on the following two reasons. First, previous studies (e.g. Grue-Sørensen and Spenser, 1988; Caveney *et al*., 2001) have found alkaloids in species closely related to the original taxon from which an alkaloid was initially described. For example, ephedrine-type alkaloids were initially described from *Ephedra distachya* (Grue-Sørensen and Spenser, 1988) and subsequently described from more *Ephedra* species as assays expanded to broader sampling within the genus (Grue-Sørensen and Spenser, 1988; Caveney *et al*., 2001). The phylogenetic reconstruction of Simmons *et al*. (2008) places the genera *Allocassine*, *Cassine*, *Lauridia* and *Maurocenia* as the sister group to *Catha edulis*, and it is proposed that species in these genera are the best candidates in which to assay for cathine and related alkaloids. The second basis for expecting the presence of cathine or similar alkaloids in other Celastraceae species is the ethnobotanical accounts of species related to qat being used as stimulants. An account from Xhosa and Khoikhoi cultures of South Africa mentions the use of *Cassine schinoides* leaves to reduce fatigue, hunger, and thirst (Watt and Breyer-Brandwijk, 1962), while an account from the Canary Islands mentions locals chewing the leaves of *Gymnosporia cassinoides* to alleviate fatigue (González *et al*., 1986).

Experimental

Chemicals, reagents and materials

Cathine and 1-phenylpropane-1,2-dione (**8**) (Fig. 1) were obtained from Sigma Aldrich (St Louis, MO), phenylethylamine (**9**), *N*-methyl-*N*-(trimethylsilyl)trifluoroactetamide and trimethylsilylimidazole were obtained from Arcos Organics (Somerville, NJ), methyl *tert*-butyl ether was obtained from Fisher Scientific (Fairlawn, NJ), and *N*-methyl-bistrifluoroacetamide was obtained from Thermo Scientific (Waltham, MA). All reagents, standards and solvents used were analytical grade. The water used in alkaloid extractions was purified using a Milli-Q (Billerica, MA) water-filtration system (water drawn off at a minimum resistivity of 18 MΩ/cm).

Plant specimens for this study (Table 1) were selected from the Celastraceae such that alkaloids were extracted from two genera in each major clade [identified from recent phylogenetic work conducted by Simmons *et al*. (2008)]. The tissue used for the analysis was from collections made as part of ongoing Celastraceae phylogenetic studies (e.g. Simmons *et al*., 2008; McKenna *et al*., 2011). All samples, with the exception of freshly harvested tissue from more distantly related species outside the Celastraceae, were fully dried in the presence of silica gel and stored over silica gel, which should preserve any cathione and cathine that is present (Chappell and Lee, 2010). Most tissue was from young but fully expanded leaves and had not been stored longer than 10 years. Lyophilised tissue from *Ephedra sinica* (Ma Huang) was obtained from a local Chinese medicine supplier.

Alkaloid extraction

The extraction protocol was adapted from Krizevski *et al*. (2007) for extracting cathinone, cathine and 1-phenylpropane-1,2-dione from fresh *Catha edulis* leaves. Leaf tissue was pulverised to a fine powder by applying high speed reciprocation for 1 min in 2 mL plastic microcentrifuge tubes containing three 3.2 mm steel beads and approximately 100 mg silica gel-dried leaf tissue. Ground leaf tissue was separated from the steel beads, accurately weighed and then transferred to glass tubes. Water (3 mL), containing 50 μg caffeine/mL as an internal standard, was added and the mixture was agitated for 45 min at room temperature (approximately 22 °C) and centrifuged at 55 r.c.f. (relative centrifugal force) for 10 min. The supernatant was removed and transferred to glass tubes, basified with 2 N sodium hydroxide (1.5 mL) and vortex-extracted for 30 s with methyl-*tert*-butyl ether (3 mL). The resulting biphasic mixture was centrifuged at 800 r.c.f. for 8 min and the organic fraction removed and placed in a new glass tube. The organic-aqueous extraction step was repeated and the combined methyl-*tert*-butyl ether-based solution was dried under a gentle stream of nitrogen gas. The entire dried plant extract was used in derivatisation.

Derivatisation

The derivatisation protocol was adapted from methods originally developed by Spyridaki *et al*. (2001) for accurately detecting ephedrine alkaloids in the urine of athletes screened in accordance with anti-doping regulations, as well as by Ranieri and Ciolino (2008), who extended this derivatisation method for accurately quantitating ephedrine alkaloids in herbal medicines containing *Ephedra sinica* and *Ephedra equisetina*. Derivatisation was completed in two stages immediately after samples were dried. The first stage of derivatisation involved adding *N*-methyl-*N*-(trimethylsilyl) trifluoroactetamide and trimethylsilylimidazole (10:1, 75 μL) and heating

Table 1. Taxa sampled with taxonomic authorities and collection information [upper case abbreviations in parentheses follow the Index Herbariorum (Thiers, 2015) acronyms and indicate the herbarium in which each specimen has been deposited, when applicable]

Family	Genus	Species	Taxonomic Authority	Collection Information
Celastraceae	*Brexia*	*australis*	G.E. Schatz & Lowry	R.H. Archer et al. 2989
	Brexia	*madagascariensis*	(Lam.) Ker Gwal.	R.H. Archer et al. 2971
	Cassine	*parvifolia*	Sond.	E. van Jaarsveld 2011–03,Western Cape, South Africa
	Cassine	*peragua*	L.	E. van Jaarsveld 2011–05, Western Cape, South Africa
	Cassine	*schinoides*	(Spreng.) R.H. Archer	E. van Jaarsveld 2011–04, Western Cape, South Africa
	Catha	*edulis*	(Vahl) Forssk. ex Endl	R.H. Archer et al. 3016, Madagascar (CS)
	Celastrus	*madagascariensis*	Loes.	R.H. Archer 379, Madagascar
	Cheiloclinium	*hippocrateoides*	(Peyr. ex Mart.) A.C.Sm.	J.A. Lombardi 6532, Brazil (HRCB)
	Crossopetalum	*gaumeri*	(Loes.) Lundell	Fairchild Tropical Garden Acc. #FTBG-941055A
	Crossopetalum	*rhacoma*	Hitchc.	M. Islam 2009–20, Bahia de Las Aguila, Domincan Republic
	Denhamia	*celastroides*	(F. Muell.) Jessup	M.W. Chase 2050, cult. Bogor, Indonesia (K)
	Elaeodendron	*xylocarpum*	DC.	M. Islam 2009-32a, Puerto Econdido, Dominican Republic
	Euonymus	*fortunei*	(Turcz.) Hand	M.P. Simmons 1913, cult. Illinois,USA (CS)
	Gyminda	*latifolia*	(Sw.) Urb.	M. Islam 2009–33, Puerto Econdido, Dominican Republic
	Gymnosporia	*buxifolia*	(L.) Szyszył.	M.P. Simmons 2429, Kenya
	Gymnosporia	*cassinoides*	Masf.	J.A. Reyes-Betancort 41716,Tenerife: Barranco de Badajoz, Canary islands
	Gymnosporia	*cassinoides*	Masf.	J. A. Reyes-Betancort TFC 49.720, Canary islands
	Gymnosporia	*cryptopetala (female)* *cryptopetala (female)* *cryptopetala (female)*	Reyes-Bet. & A. Santos	S. Scholz 39426, Fuerteventura, Canary Islands
	Gymnosporia	*cryptopetala (male)* *cryptopetala (male)* *cryptopetala (male)*	Reyes-Bet. & A. Santos	S. Scholz 41096, Fuerteventura, Canary Islands
	Gymnosporia	*divaricata*	Baker	R.H. Archer et al. 2891, Madagascar (CS)
	Gymnosporia	*gracileps*	Loes.	M.P. Simmons 2237, Ethiopia
	Gymnosporia	*heterophylla*	Loes.	M.P. Simmons 2075, Ethiopia
	Gymnosporia	*linearis*	(L.f.) Loes.	R.H. Archer et al. 2935, Madagascar (CS)
	Gymnosporia	*senegalensis*	Loes.	M.P. Simmons & G.W. Ngugi 2387, Kenya (CS)
	Gymnosporia	*senegalensis*	Loes.	R.H. Archer et al. 3039, Madagascar (CS)
	Halleriopsis	*cathoides*	R.H. Archer (ined.)	R.H. Archer et al. 3035, Madagascar (CS)
	Hylenaea	*comosa*	(Sw.) Miers	J.A. Lombardi 6400, Brazil (HRCB)
	Lauridia	*tetragona*	(L.f.) R.H.Archer	E. van Jaarsveld 2011–01, Western Cape, South Africa
	Lophopetalum	*arnhemicum*	Byrnes	W. Price s.n., Australia (BRI)
	Maurocenia	*frangula*	Mill.	E. van Jaarsveld 2011–02,Western Cape, South Africa
	Maytenus	*grenadensis*	Urb.	M. Islam 2009–51, Grenada
	Maytenus	*boaria*	Molina	M.P. Simmons & G. Smick 1908, cult. San Francisco, California (CS)
	Microtropis	*fokienensis*	Dunn	Xiong Weizhong229, China (MO)
	Microtropis	*triflora*	Merr. and Freeman	H. He 09, Chongqing, China (CTC)

(*Continues*)

Table 1. *(Continued)*

Family	Genus	Species	Taxonomic Authority	Collection Information
	Mystroxylon	*aethopicum*	(Thunb.) Loes.	R.H. Archer et al. 2921, Madagascar (CS)
	Peripterygia	*marginata*	Loes.	M.P. Simmons 1793, New Caledonia (BH)
	Pleurostylia	*opposita*	(Wall.) Merr. & F.P. Metcalf	A. Ford 2318, Australia (BRI)
	Polycardia	*libera*	O. Hoffm	R.H. Archer et al. 2899, Madagascar (CS)
	Polycardia	*phyllanthoides*	Lam.	R.H. Archer et al. 2904, Madagascar (CS)
	Pseudocatha	*mandenensis*	ined.	R.H. Archer et al. 2996, Madagascar (CS)
	Salacia	sp. nov. (?)	not applicable	R.H. Archer et al. 3040, Madagascar (CS)
	Salaciopsis	*glomerata*	Hürl.	M. P. Simmons 1895, Sud Province, New Caledonia
	Schaefferia	*fructescens*	Jacq.	Fairchild Tropical Garden Acc. #72611
	Siphonodon	*pendulus*	F.M. Bailey	A. Ford 4529, Australia (BRI)
	Xenodrys	*micranthum*	R.H. Archer (ined.)	R.H. Archer et al. 2902, Madagascar (CS)
	Zinoweieia	*matudai*	Lundell	M.P. Simmons & G. Smick 1906, cult. San Francisco, California (CS)
Ephedraceae	*Ephedra*	*sinica*	Stapf	L.R. Tembrock, lyophilised powder purchased as Ma Huang
Papaveraceae	*Macleaya*	*cordata*	R.Br.	Denver Botanic Gardens Acc. # 0.02386
Rubiaceae	*Galium*	*aparine*	L.	L.R. Tembrock 11–003, cult. Colorado, USA
Salicaceae	*Populus*	*angustifolia*	James	L.R. Tembrock 11–004, cult. Colorado, USA
	Populus	*tremuloides*	Michx.	L.R. Tembrock 11–005, cult. Colorado, USA

for 15 min at 80 °C. Then *N*-methyl-bistrifluoroacetamide (30 μL) was added and again heated at 80 °C for 15 min.

GC–MS conditions and instrumentation

Experiments were performed using a Thermo GC Trace Ultra coupled with a Thermo DSQ II mass spectrometer. The column used was a Thermo 30 m TG 5 MS with an internal diameter of 0.25 mm and a film thickness of 0.25 μm. Helium was used as the carrier gas at a flow rate of 1.2 mL/min. The injection-port temperature was 250 °C using split mode at a ratio of 1:10. The GC-oven regime for each injection was 80 °C for 2 min, then ramped at 5 °C/min until 190 °C was reached, followed by ramping at 15 °C/min until 310 °C was reached and then held for 1 min. The mass spectrometer was operated using electron ionisation at 70 eV and scanned in positive mode between *m/z* 50–650 from 4 to 20 min. Cathine and phenylethylamine identifications were determined by matching retention times and mass spectra of authentic standards (Sigma Aldrich, St Louis, MO).

Method validation

For **3**, **5**, **8** and **9** (Fig. 1), the primary compounds of interest, linearity across a dilution series (25, 2.5, 0.25 μg) limit of detection, and the limit of quantitation were experimentally verified. The samples were prepared separately for each compound and dilution step. The extraction, derivatisation and instrumentation procedures for the dilution series were the same as the methods for leaf material. This was done to ensure that the standards were recoverable through the complete processing protocol. Limit of detection and limit of quantitation were determined through serial dilutions at 3× and 10× signal-to-noise. The cathinone standard could not be verified because its' possession and use for experimental purposes requires a schedule I Drug Enforcement Agency permit.

Repeatability of extraction and derivatisation was determined by replicating all steps twice across each species sampled and examining variability between signal peaks. Repeatability and stability for cathine extraction and derivatisation from plant material was tested from seven different leaf samples.

Data analysis and statistics

For each sample, raw data files were converted to .cdf format and a matrix of molecular features as defined by retention time and *m/z* was generated using the XCMS package in R (v1.42.0, Smith *et al.*, 2006) for feature detection and alignment. Raw peak areas were normalised to total ion signal in R, outlier injections were detected based on total signal and principal component 1 of PCA (principal component analysis), and the mean area of the chromatographic peak was calculated from duplicate injections. Molecular features were clustered using RAMClustR (Broeckling *et al.*, 2014). Metabolites were annotated by searching mass spectra from the RAMclustR-derived clusters against the National Institute for Standards and Technology (NIST v14, nist.gov), Massbank (http://www.massbank.jp) and Golm MS databases (http://gmd.mpimp golm.mpg.de) for metabolite identification (e.g. Heiling *et al.*, 2016) For all annotated compounds of interest, peak detection and relative quantitation (using peak area) were conducted from the original dataset using Tracefinder v3.2 (Thermo Scientific, Waltham, MA). PCA was conducted on mean-centred and univariate-scaled data using SIMCA P+ v12 (Umetrics, Umea, Sweden). Patterns of compound abundance and distribution were examined in the PCA by observing individuals that were plotted outside the 95% confidence interval of Hotelling's T^2 (Wilkström *et al.*, 1998). From these patterns, PCA loadings were examined to determine which compounds were providing signal for the outlier species (e.g. Davis *et al.*, 2013).

Results and discussion

Derivatisation

A two-stage derivatisation method was used to reduce the undesirable production of multiple derivatives or the lack of separation among norephedrine and similar compounds and their isomers. *N*-Methyl-*N*-(trimethylsilyl)trifluoroactetamide trimethylsilylates amides, secondary amines, hydroxyls and hydrogen on polar compounds with application to a broad range of compounds especially steroid hormones (Shareef *et al.*, 2006). Trimethylsilylimidazole trimethylsilylates carbonyl, steroid and secondary alcohols and

are commonly used in multiderivatisation schemes (e.g. Jyske *et al*., 2015). *N*-Methyl-bistrifluoroacetamide trifluoroacetylates alcohol and certain carbohydrate functional groups are commonly applied to amphetamine detection (e.g. Hidvégi *et al*., 2008).

Previous work (Donike, 1975; van der Merwe and Hendrikz, 1995) has shown that the *N*-methyl-*N*-(trimethylsilyl) trifluoroactetamide/*N*-methyl-bistrifluoroacetamide method commonly used to detect ephedrines produced multiple derivatives. Therefore this investigation used *N*-methyl-*N*–(trimethylsilyl) trifluoroactetamide/trimethylsilylimidazole in the first step to reduce the production of multiple derivatives among the target compounds by not trimethylsilylating amine groups (Spyridaki *et al*., 2001). Trimethylsilylimidazole is often complexed with the catalysing reagent methyltrichlorosilane, but from testing during the course of this study it was found that trimethylsilylimidazole with methyltrichlorosilane produced multiple derivatives and thus pure trimethylsilylimidazole was used (Popl *et al*., 1990). Molnár *et al*. (2016) also found that derivatisation reagents containing catalysts produced unstable derivatives with cathinone. Subsequently, *N*-methyl-bistrifluoroacetamide was used in the second separate step to trifluoroacetylate amino groups.

Detection of cathinone, cathine and related compounds

Detection of cathine (**3**) was linear over the range 25 μg to 0.025 μg (R^2 = 0.999). The limit of detection and limit of quantitation for cathine were 0.2 μg/mL and 0.8 μg/mL, respectively. Cathine was not detected in any species assayed (Table 1) other than *Catha edulis* [Figs 2, 3(B), 3(F)]. Given these results and the extraction and sample preparation protocol used, the limit of detection and limit of quantitation were estimated to be 0.03 and 0.1 μg cathine/100 mg of dry leaf material, respectively. Across the six replicate samples of qat an average of 7.6 μg/100 mg of tissue (dry weight) was detected.

The amine group of cathinone (**1**) is expected to be triflouroactetylated and have prominent peaks at *m/z* 51 (10%), 77 (38%), and 105 (100%) in its electron ionisation MS (F. Westphal, pers. Comm., 2011; Dell'Acqua *et al*., 2013). Cathinone was not detected from the silica gel-dried samples of *Catha edulis*, which is attributed to the older age of the leaves when harvested. Molnár *et al*. (2016), despite using a sensitive direct derivatisation process, could not detect cathinone from freeze dried qat leaves. They attributed the lack of detectable cathinone in the leaf material to the growth conditions of the plant. Additionally none of the proposed precursors or end products (except cathine) in the cathinone biosynthesis pathway, as proposed by Krizevski *et al*. (2010), were found in species other than *Catha edulis*. The immediate precursor of cathinone, 1-phenylpropane-1,2-dione (**8**; Krizevski *et al*., 2007), was only previously detected at very low concentrations in *Catha edulis* and *Ephedra* spp., was not detected in the *Catha edulis* or *Ephedra sinica* samples in this study. The limit of detection for standard 1-phenylpropane-1,2-dione with the methods used in this study was 1.6 μg/mL.

If cathine is further metabolised in other Celastraceae species, ephedrine (**6**) and pseudoephedrine (**5**) are the next products resulting from the action of *N*-methyltransferase (Okada *et al*., 2008; Krizevski *et al*., 2010). In the case of **5**, the derivatisation method used produced a single peak at 8.18 min. The derivatisation method was verified for ephedrine alkaloids using both a pseudoephedrine standard and lyophilised tissue of *Ephedra sinica*. Both **5** and **6** where clearly detected in the Ma Huang preparation of *Ephedra sinica* but none of the other phenylpropylamino alkaloids described in Krizevski *et al*. (2010). The lack of ephedrine precursors reflects the results of Krizevski *et al*. (2010) in which fresh young stems were the only plant parts found to possess **8** and **1**. In accord with earlier reports of HPLC-based analysis (Szendrei, 1980), no Celastraceae species in this study, including *Catha edulis*, was found to contain detectable amounts of pseudoephedrine (**5**) or ephedrine (**6**).

Much of the plant material used in this study was from fully expanded leaves. Therefore, if the pathway in other Celastraceae species is the same as that of *Catha edulis*, the expectation is that cathine (**3**) might be the most abundant stimulant alkaloid detectable in older leaves. Furthermore, if **3** was detected, then the presence of cathinone (**1**) could be inferred in the younger leaves. Chappell and Lee (2010) demonstrated that **1** and **3** could still be detected from air-dried leaves after three years stored without any desiccant at room temperature, and speculated that cathinone (**1**) and cathine (**3**) could remain detectable after 10 years stored at room temperature. Nearly all of the plant material used in this present study was less than 10 years old at the time of analysis and had been stored over silica gel at room temperature throughout that time. Therefore the age and condition of plant material was not a major factor in the observed absence of **3** from other species.

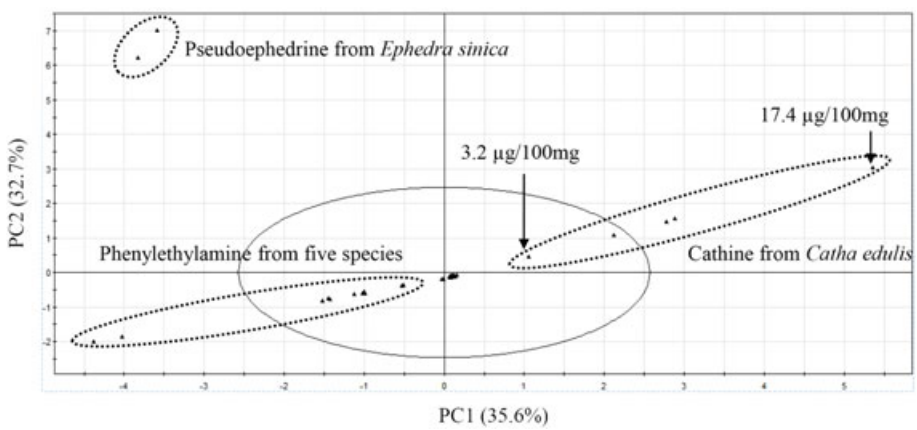

Figure 2. Principal component analysis (PCA) plot with all species assayed using signal from the phenylalanine alkaloids cathine, phenylethylamine and pseudoephedrine as variables. Dashed ellipses group samples containing one of three alkaloids and the solid ellipse indicates Hotelling's T^2 at 0.95. Points clustered at the centre are all samples testing negative for any of the three alkaloids. [Colour figure can be viewed at wileyonlinelibrary.com]

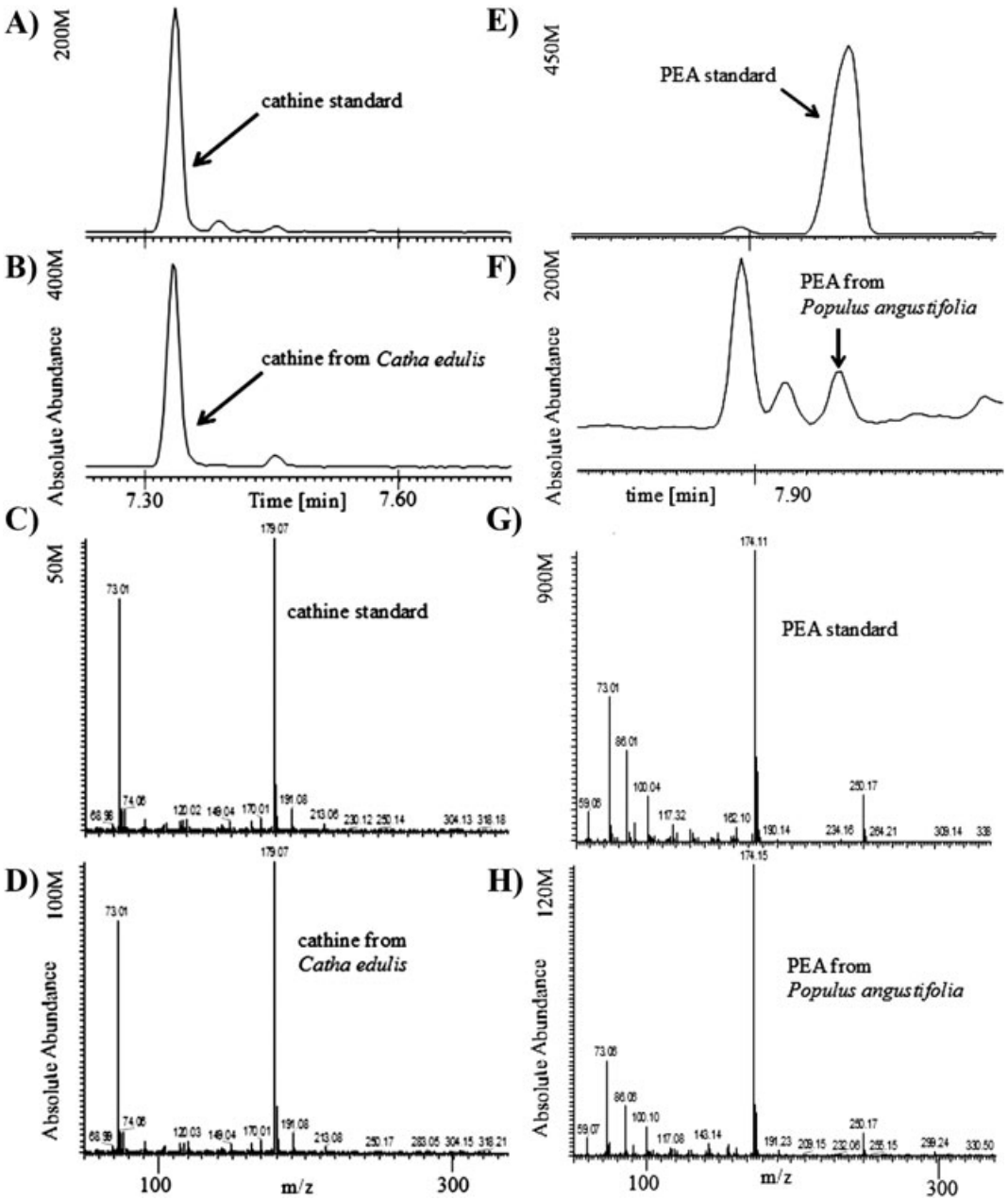

Figure 3. Chromatograms comparing (A) cathine standard, to (B) extract from *Catha edulis*, and (E) phenylethylamine standard, (F) extract from *Populus angustifolia*. The electron ionisation mass spectra of (C) cathine standard, (D) extract from *Catha edulis*, (G) phenylethylamine standard, and (H) extract from *Populus angustifolia*. 'M' on the *y* axis is an abbreviation for million. All figures indicate the derivatised molecules.

It is not unexpected for only a single species within a lineage to synthesise a given alkaloid, as is the case with morphine from *Papaver somniferum* (Ziegler *et al*., 2009). In the case of *Papaver somniferum*, the closely related species *Papaver bracteatum* and *Papaver arenarium* produce the precursors to morphine but not morphine itself (Ziegler *et al*., 2009). Given that only three enzymatic steps are needed to produce cathine from benzoic acid, it is possible that the entire pathway evolved after *Catha edulis* diverged from its most recent common ancestor with the extant sister group of *Allocassine*, *Cassine*, *Lauridia*, and *Maurocenia*. But it cannot be ruled out that a species which is closely related to *Catha edulis* might have the ability to synthesise cathine or related alkaloids given that only five (*Cassine parvifolia*, *Cassine peragua*, *Cassine schinoides*, *Lauridia tetragona* and *Maurocenia frangula*) of the 13 species from the sister group were sampled. *Catha edulis* is the only species currently recognised in *Catha* (Robson *et al*., 1994; Archer and van Wyk, 1997; Simmons *et al*., 2008).

Additional compounds detected by GC–MS

While the extraction and derivatisation methods were expected to detect alkaloids, including otherwise non-volatile alkaloids, numerous other compounds were also detected. A signal detection workflow resulted in the detection of 296 feature clusters (defined as a cluster of *m/z* at a single retention time) from the dataset after alignment and clustering. In order to find compounds unique to a

single species or clade, principal component PCAs were conducted across the sample set. The PCAs were organised by taxonomic grouping in order to find compounds that varied significantly across different levels of biological organisation (family and genus). Using this approach 27 compounds were detected (Fig. 4). Additional compounds were characterised in the dataset but could not be assigned a biological origin and were therefore excluded from further analyses due to low signal strength in the chromatogram, lack of repeatability across extractions and/or low quality ion-spectrum matches. All 27 compounds were found to be significantly greater than the average background signal (MS "noise"). This processing and discovery method was confirmed by the detection of cathine and phenylethylamine based on matching spectra and retention times to authentic standards.

Discovery of phenylethylamine in Celastraceae species

Phenylethylamine (**9**) [Figs 1–3(G), 4] was found in *Brexia madagascariensis*, *Crossopetalum rhacoma*, *Lauridia tetragona*, *Polycardia liberia*, and an outgroup species, *Populus angustifolia*, but not in *Catha edulis*. The GC retention time and electron ionisation mass spectrum for phenylethylamine found in these species was confirmed by comparing to an authentic standard [Fig. 3(E)–3(H)] that was processed through the extraction, derivatisation, and GC–MS protocols.

Phenylethylamine (**9**) is similar to cathine (**3**) in that few enzymatic steps are needed to produce phenylethylamine from phenylalanine or tyrosine (Smith, 1989). Therefore the dispersed pattern of phenylethylamine presence in Celastraceae and other plant lineages indicates that it is a non-specific product of plant metabolism. When optimised onto (Fitch, 1971) the most recent Celastraceae phylogeny (Simmons *et al.*, 2008; McKenna *et al.*, 2011) the presence of **9** shows no evident relationship to the cathine pathway (Fig. 4) given that there are four inferred independent origins of phenylethylamine. Further supporting this interpretation is the result from Pagel's (1994) phylogenetic test of correlated character evolution, in which no significant correlation was found between **3** and **9**.

Annotation of sterols, terpenes, and related compounds

While the extraction and derivatisation protocols targeted alkaloids, 27 other compounds with a discernable signal were found in the dataset and thus characterised. Of the 27 compounds detected from our workflow, 18 were annotated as sterol-like, eight were annotated as terpene- or terpenoid-like, and one did not match well to any compound type in the metabolite databases used. A wide diversity of terpenes, including numerous unique types, have been described from the Celastraceae (Shan *et al.*, 2013). The bioactivity of terpenes in the Celastraceae has been studied within certain species given their potential application as

		Stimulant Alks.		Sterol Annotations																	Terpenes/Terpenoids Annotations								?
Phylogenetic Relationships	Species	Cathine	PEA	12	12.2	12.9	13	13.2	13.3	13.3	14.1	15.2	15.7	15.9	17.7	18.1	18.2	18.3	18.4	19	20.1	18	18.5	18.5	18.7	18.9	19.6	19.8	22.3	19.1
	Microtropis triflora	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Microtropis fokiensis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Zinowiewia matudai	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Euonymusfortunei	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Celastrus madagascariensis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Gyminda latifolia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Schaefferia frutescens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Lauridia tetragona	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Lauridia reticulata	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Cassine schinoides	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	+	++	++	++	++	-	++
	Cassine peragua	-	-	+	-	-	-	-	-	-	+	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	Maurocenia frangula	-	-	++	++	++	++	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	Catha edulis	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Gymnsporia cassinoides	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-
	Gymnsporia linearis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Gymnsporia senegalensis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Mystroxylon aethiopicum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Halleriopsis cathoides	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	+	-	-	-	-	-	-
	Pleurostylia opposita	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Pseudocatha mandenensis	-	-	-	-	+	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	++	-
	Elaeodendron xylocarpum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brexia australis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brexia madagascariensis	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Polycardia liberia	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Polycardia phyllanthoides	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Lophoeptalum arnhemicum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Hylenea comosa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Cheiloclinium hippocrateoides	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-
	Salaciopsis glomerata	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Maytenus disticha	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Maytenus boaria	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	Xenodrys micranthum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Crossopetalum gaumeri	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Crossopetalum rhacoma	-	++	-	-	-	-	-	-	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-
	Peripterygia marginata	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	+	-	-	-	-
	Denhamia celastroides	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Additional Species Sampled	*Cassine parviflora*	-	-	-	-	+	++	-	-	-	++	-	-	-	++	++	++	-	++	-	-	++	-	+	-	+	+	-	++	-
	Gymnosporia buxifolia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-	++
	Gymnosporia heterophylla	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-
	Gymnosporia wallichiana	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-	+	+	-	-	-
	Populus angustifolia	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Populus tremuloides	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-

Figure 4. Phylogenetic relationships among the Celastraceae taxa sampled (Simmons *et al.*, 2008; McKenna *et al.*, 2011) with cathine, phenylethylamine, and feature clusters annotated as sterols or terpenes/terpenoids. Occurrences of cathine and phenylethylamine are mapped onto the tree using Fitch (1971) optimisation; bold branches on the tree represent independent derivations. The symbol ++ indicates a significant signal compared to background across multiple extractions and injections, + indicates possible presence of the compound but not significant across both extractions and/or injections, and - indicates a signal that is indistinguishable from the background across both extractions and injections. Annotated compounds are labelled by retention time in minutes.

painkillers, cancer treatments, anti-HIV agents, diabetes treatments, and antidepressants (Shan *et al*., 2013).

It was found that *Pseudocatha mandenensis*, *Maurocenia frangula*, *Cassine parviflora,* and *Halleriopsis cathoides* were enriched in the type and/or quantity of compounds annotated as sterols (Fig. 5). Four compounds annotated as sterols were highly abundant in *Pseudocatha mandenensis*, three in *Maurocenia frangula*, one in *Cassine parviflora,* and one in *Halleriopsis cathoides* (Figs 4, 5). Seven compounds annotated as sterols of relatively high abundance were detected in two or more of the following three species: *Pseudocatha mandenensis*, *Maurocenia frangula* and *Cassine parviflora* (Figs 4, 5). Sterols are routinely discovered in phytochemical assays (e.g. Kaweetripob *et al*., 2013) of Celastraceae. However, because *Pseudocatha mandenensis*, *Maurocenia frangula* and *Cassine parviflora*, have not previously been assayed for sterols the compounds annotated here maybe undescribed molecules. Additional experiments are needed to confirm the initial annotations and/or describe these potentially novel compounds. The sterol-type compound from *Halleriopsis cathoides* is of particular interest for the following three reasons: (1) *Halleriopsis cathoides* has not been formally published taxonomically, thus it has almost certainly never been assayed; (2) the annotated compound [retention time (Rt) = 20.1 min] is unique across the entire dataset; (3) the highest match was to preganalone, a neurosteroid with sedative, anxiolytic, anaesthetic, and anticonvulsant properties (Carl *et al*., 1994). These conclusions regarding sterols from the present sample set are provisional but provide a sound starting point for further research into the molecular structure and possible bio-activity of these compounds.

Eight compounds annotated as terpene-like with sufficient signal to be distinguished from background [signal-to-noise ratio

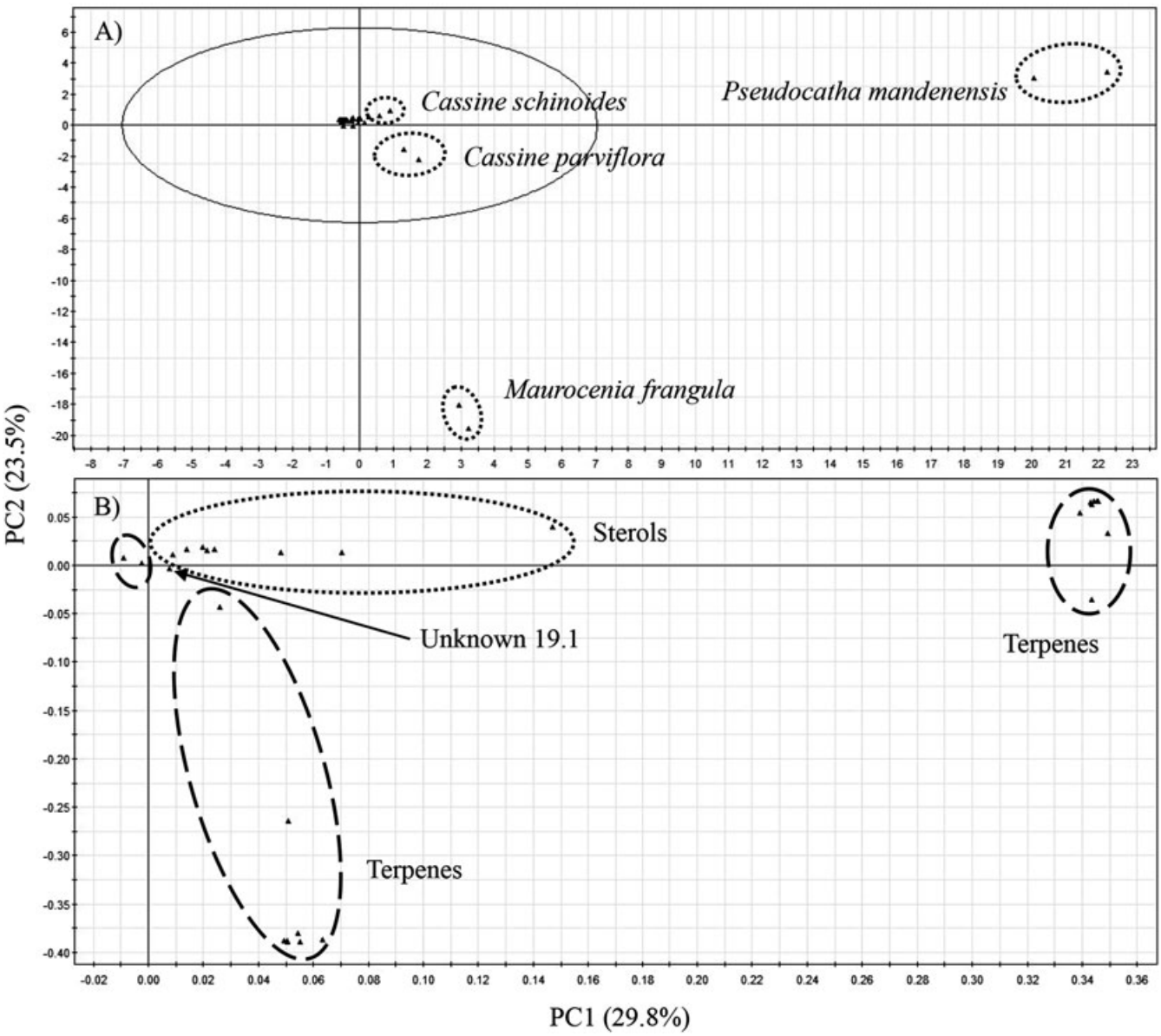

Figure 5. (A) Principal component analysis (PCA) with all species assayed using signal from the 27 annotated compounds. The dashed ellipses indicate samples enriched for type and quantity of annotated compounds and the solid ellipse indicates Hotelling's T^2 at 0.95. (B) Loading scores for the annotated compounds used in PCA indicate the strength of signal contributed by these compounds. Long dashed ellipses group sterols and short dashed ellipses group terpenes.

(S/N) >3] and annotated were found in the dataset. Five compounds annotated as terpenes were found in high abundance in *Cassine schinoides*, one in *Pseudocatha mandenensis*, one in *Peripterygia marginata,* and one found in both *Pseudocatha mandenensis* and *Cheiloclinum hippocratedides* (Figs 4, 5). As with sterols, terpenes are commonly found in the Celastraceae (Shan *et al*., 2013). It appears that the species *Cassine schinoides*, *Pseudocatha mandenensis,* and *Cheiloclinium hippocrateoides* have not been part of any published phytochemical work on terpenes. The most interesting finding regarding the terpene annotations is the five compounds annotated as terpenes that were found in significant abundance in *Cassine schinoides.* While terpenes and triterpenoids have been described from more distantly related species such as *Elaeodendron balae* (Fernando *et al*., 1989) and *Elaeodenron xylocarpa* (Núñez *et al*., 2013; formerly *Cassine*), no phytochemical assay has been conducted on the more narrowly defined southern African *Cassine* genus, which is distantly related to *Elaeodendron* (Simmons *et al*., 2008). Two annotations for terpenes occurring in *Cassine schinoides* were friedelanone, a triterpene type broadly diversified in the Celastraceae and found in *Elaeodendron balae* (Fernando *et al*., 1989). While friedelanones have not been found to be stimulants, little is known about the pharmacology of most of the friedelanones discovered from the Celastraceae (Shan *et al*., 2013). Triterpenes from *Maytenus gonoclada* and *Maytenus imbricata* were found to be central nervous system stimulants through inhibition of acetylcholinesterase (Rodrigues *et al*., 2014). Furthermore, some terpenoids such as tetrahydrocannabinol can have pronounced pharmacological action (Isbell *et al*., 1967). Whether the terpenes in *Cassine schinoides* have stimulant effects is not known, but the diversity and abundance of these compounds in *Cassine schinoides* relative to other species warrants further research. Additionally, given that *Pseudocatha mandenensis* and *Cheiloclinium hippocratedides* have not been previously assayed, the terpene annotations herein require further clarification.

The clade of *Cassine* + *Maurocenia* shows a pattern of terpene and sterol enrichment with *Cassine schinoides* enriched in terpenes and the clade *Cassine peragua* + *Maurocenia frangula* enriched in sterols. This distribution pattern indicates a possible stepwise evolutionary pattern in this clade as terpenes are precursors to sterols (e.g. Pandit *et al*., 2000; Niehaus *et al*., 2011).

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