Full title: Seasonal Analysis of the Tropane Alkaloid Ecgonine Methyl Ester and the Occurrence of other Highly Valued Tropanes in the South African *Erythroxylum* Trees

Short title: Seasonal Variation and Detection of Highly Valued Tropane Alkaloids in the South African Coca Trees

P. Sewes F. Alberts ¶[1], Marelize Daneel ¶[1], Adriaan A.S. Marais ¶[2], Denis A. Baranenko ¶[3] J.J. Marion Meyer[1,3] ¶*

[1]Department of Plant and Soil Sciences, University of Pretoria, Pretoria, South Africa

[2]Forensic Science Laboratory: Section Chemistry, South African Police Service, Pretoria, South Africa

[3]ITMO University, Saint-Petersburg, Russia

* Corresponding Author

E-mail: marion.meyer@up.ac.za (JJM)

Abstract

The coca family (Erythroxylaceae) consists of trees and shrubs sub-divided into four genera: *Aneulophus, Nectaropetalum, Pinacopodium,* and *Erythroxylum*, which include species with highly valuable medicinal compounds. *E. delagoense, E. emarginatum,* and *E. pictum* are endemic to southern Africa and have great pharmaceutical potential based on their traditional uses. Previous studies have shown certain inconsistencies in terms of the presence or absence of tropane alkaloids in these species, resulting in a need for further research and clarification. Therefore, the aim of this study was to determine the seasonal variation of the immediate biosynthetic precursor of cocaine, the tropane alkaloid, ecgonine methyl ester in the three South African *Erythroxylum* species by means of gas chromatography – mass spectrometry, as well as to conduct a phytochemical screening for observing the presence of other potential compounds and tropane alkaloids. We found significant differences in tropane concentrations from the seasonal variation study, explaining the discrepancies in previous reports on its presence/absence in these species. Furthermore, we report for the first time on the occurrence of selected highly valuable tropane alkaloids in *E. emarginatum* currently used in 'blockbuster-medicine'.

Keywords: *Erythroxylum*; ecgonine methyl ester; tropane; alkaloid; GC-MS; phytochemical.

Introduction

The family Erythroxylaceae consists of four genera (*Aneulophus, Nectaropetalum, Pinacopodium,* and *Erythroxylum*) including approximately 240 species of flowering trees and shrubs (Stevens 2015). The *Erythroxylum* species are well known for their alkaloid content and are especially famous for their cocaine and other tropane alkaloid producing members (Chin et al. 2006; De Simone et al. 2008; Oliveira et al. 2010). Besides tropane and other alkaloid derivatives, there have been reports of diterpenoids, triterpenoids, flavonoids, and tannins in some species of the genus (Evans 1981; Bohm et al. 1988; Ansell et al. 1993; Chin et al. 2006). Limited research has been published on the chemical profile of the endemic South African species, the African- (*E. emarginatum* Thonn.), forest- (*E. pictum* E.Mey. ex Harv. & Sond), and small-leaved coca trees (*E. delagoense* Schinz.) (Nishiyama et al. 2007).

There are no studies with conclusive reports on the absence or presence of cocaine, while only a few studies have been done on other medicinally valuable compounds and their precursors in the southern Africa *Erythroxylum* species. Previous studies by Nishiyama et al. (2007) have found the presence of anhydrous methyl ecgonine (also known as ecgonidine methyl ester), a cocaine precursor, in the stem-bark and twigs of *E. emarginatum*, although the presence of this compound was not detected in the leaves. Furthermore, the study conducted by Evans (1981) did not report the presence of any alkaloids in *E. delagoense.* Both these findings contrast with the results reported in the current study.

The reported traditional uses of the southern African *Erythroxylum* species might indicate the presence of tropane and other related alkaloids, as they include treatments for a variety of pain and stimulatory related ailments (Lounasmaa and Tamminen 1993; Nishiyama et al. 2007; Grynkiewicz and Gadzikowska 2008; Corrigan et al. 2011). These traditional uses emphasise the need for further research on the southern Africa varieties.

Limited studies have been done on the optimal extraction temperature with regards to the cocaine precursor and related tropane alkaloids when pressurised liquid extractions (PLE) are used. The study conducted by Moroczek et al. (2006) showed that the extraction temperature of 90 °C and 110 °C at 100 bar is highly efficient for extracting scopolamine (1 % tartaric acid in methanol) and hyoscyamine (methanol) from *Datura* species, respectively. Whereas the study conducted by Brachet et al. (2001) showed that 80 °C at 200 bar using methanol are the optimal conditions for extracting cocaine and benzoylecgonine from dried *E. coca* leaves. These reports do not give a clear indication of the optimal extraction temperature regarding cocaine precursors and related alkaloids.

The aims of this study were to establish a general phytochemical profile for the three South African *Erythroxylum* species, and investigate whether seasonal variation of ecgonine methyl ester may explain the discrepancies seen in previous reports on the presence/absence of this tropane alkaloid in the South African *Erythroxylum* species. Moreover, a tropane alkaloid screening and a temperature based PLE methodology analysis were performed, using gas chromatography – mass spectrometry (GC-MS).

Materials and Methods

Collection and extraction of plant material for seasonal analysis

Leaf material from three *Erythroxylum* species were collected at the Lowveld National Botanical Garden situated in Mbombela (Nelspruit), Mpumalanga. The material was harvested from the northern side of the trees between 11:00 am and 13:00 pm. Voucher specimens of *E. delagoense* (PRU # 121564), *E. emarginatum* (PRU # 121566), and *E. pictum* (PRU # 121565) were deposited in the H.G.W.J. Schweickerdt Herbarium, University of Pretoria. Plant material used in the tropane alkaloid screening was collected on 18 September 2014 (spring) and 17 April 2015 (autumn). Plant

material used for the seasonal variation study was collected on 14 July 2015 (winter), 08 October 2015 (spring), 26 January 2016 (summer), and 13 April 2016 (autumn). Preservation of the leaf material was done by means of dehydration using a freeze-dryer (VirTis, United Scientific) and storing at 5 °C. The plant material was collected in accordance with the Department of Environmental Affairs (BABS/000515N) and the South African National Biodiversity Institute (SANBI).

Crude leaf extracts were prepared using a Büchi E-916 Speed-Extractor. The PLE parameters were as follows: five replicates of dried leaf material were placed in 40 mL stainless steel tubes, the extraction was performed using pressurised methanol at 100 bar and 60 ºC. Each extraction consisted of four cycles, each including a 1 min heating phase, 9 min solvent holding phase, and a discharge phase of 5 min. At the end of each cycle, a nitrogen gas purge was performed for 8 min, with a total extraction time of 1 h and 32 min. All the extracts were dried using a Büchi Genevac (EZ-2 plus) at 40 °C, and stored at 5 °C until further analysis.

Phytochemical profile

A general phytochemical profile analysis for alkaloids, cardiac glycosides, flavonoids, phenolics, saponins, tannins, and terpenoids was carried out. Crude extracts of *E. delagoense, E. emarginatum* and *E. pictum* (samples collected in summer 2016) were used. The analysis was done as described by Mushtaq et al. (2014) with the modification of using zinc metal powder instead of zinc turnings in the test for flavonoids.

Alkaloid extractions

Two groups of alkaloid extractions were prepared. Group one included *E. delagoense, E. emarginatum,* and *E. pictum* (autumn 2015 collection), used in the tropane alkaloid screening, whereas group two consisted of *E. emarginatum* (spring 2014 collection) and was used for the PLE

methodology analysis. Crude extracts of the two groups were prepared in the same manner as described above, however, different extraction temperatures were applied to group two. The temperature modifications included: 30 °C, 40 °C, 60 °C, and 80 °C. After obtaining and drying the crude extracts in a Genevac, a liquid-liquid extraction method described by the United Nations Office on Drug and Crime (UNODC) was used to obtain the alkaloid extracts (Turner et al. 1981; United Nations Office on Drugs and Crime 2012). The extracted alkaloids were stored at 5 ºC until further analysis.

GC-MS analysis

Five replicates per species (1 mg extract/mL) were prepared in distilled methanol (dMeOH) (Merck, South Africa) and analysed on an Agilent 7890A GC linked to a 5975 – mass selective detector (MSD) spectrometer with an electron impact (EI) mode at 70 eV. The compounds were separated using a DB – 5 ms (30 m x 0.25 mm, 0.25 µm film thickness) capillary column with helium as carrier gas. Splitless injections of 2 µL were performed, with both the injector and detector temperature set at 300 °C. The oven program was set at an initial temperature of 50 °C held for 1 min, thereafter the temperature was increased to 320 °C at a rate of 60 °C per min and it was held for 6 min and 30 sec, making a total run time per sample of 12 min. Acquisition of mass spectral data was set at a mass range of 35.0 to 550.0 *m/z,* with a *m/z* threshold of 10. The chromatograms and spectra were analysed using the Enhanced ChemStation software (MSD ChemStation E.02.02.1431) provided by Agilent Technologies, Inc. (Agilent Technologies, Inc., USA). The statistical analysis was performed using GraphPad Prism version 4 (GraphPad Software, Inc., 2005).

Quantification was done by constructing an ecgonine methyl ester standard (Cerilliant, USA) calibration curve, using concentrations of 0.0 µg/mL, 1.0 µg/mL, 2.0 µg/mL, and 5.0 µg/mL. Identification and confirmation of ecgonine methyl ester in the standards, as well as the crude extracts, were done by extracting the 82, 96, and 199 *m/z*-ions, in the selected ion monitoring (SIM) mode of

the Enhanced ChemStation software. The specific mass-ions were selected as identifier ions based on literature and their prominence in the respective standard samples (Jenkins et al. 1996; Smith and Casale 2010). The calibration curve was therefore set up by analysing the GC chromatograms in SIM mode and manually integrating the average analyte area of the 96 *m/z*-ion peaks. The same 96 *m/z*-ion was analysed during SIM mode for each respective sample and related back to the standard curve for quantitative analysis. For further confirmation, the mass-ion peak abundance and fragmentation pattern of the ecgonine methyl ester was correlated back to standard spectra in the NIST (National Institute of Standards and Technology) version 14 (version 2.2, Agilent Technologies, USA) software, with a ≥ 90 % similarity index (SI). This final confirmation step was also used in the screening analysis for other related tropane alkaloids in the respective alkaloid extracts.

Results and Discussion

To improve our understanding of the effect seasonal variation could have on the identification and reporting of tropane alkaloids in the South African *Erythroxylum* species, ecgonine methyl ester was quantified during each climatic season (Fig. 1). Since ecgonine methyl ester is the immediate precursor in cocaine biosynthesis and the most abundant tropane alkaloid found during a pilot study in the South African species, it was chosen for quantification. It should be noted that when methanol is used as a solvent during GC-MS analysis of ecgonine, it frequently converts into two isomers, ecgonine methyl ester and pseudoecgonine methyl ester, resulting in the observation of two peaks on the GC chromatogram, as shown by Casale (1992). This was evident in the standard as well as extracted samples. In this study, each isomer peak was integrated separately and the representative analyte areas summed to calculate the total quantity of ecgonine methyl ester present.

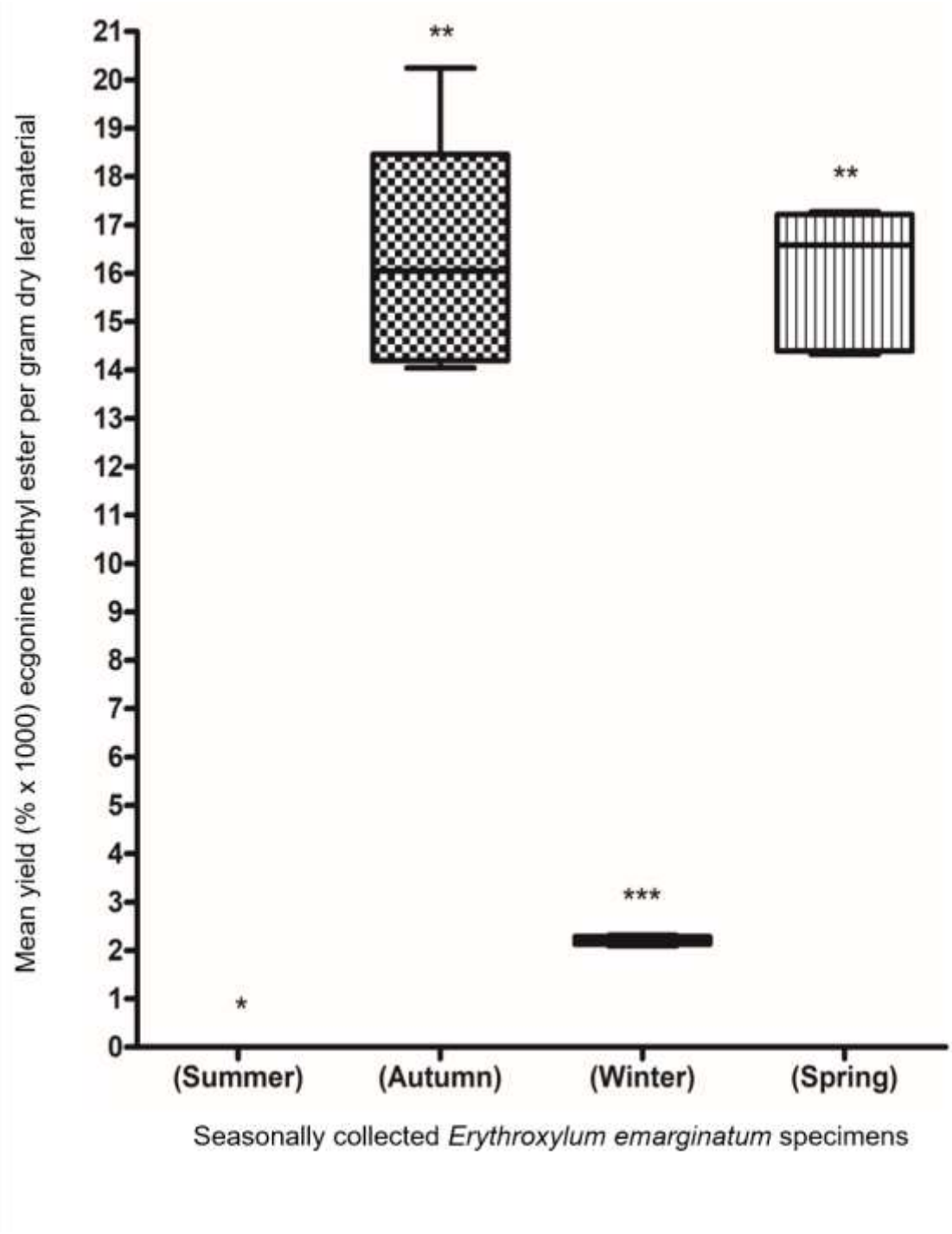

Fig. 1 The *E. emarginatum* seasonal average yield (% x 1000) ecgonine methyl ester per gram dry leaf material. The specimens with the same number of asterisks (*) do not differ significantly at a 95 % confidence level

We report here only on *E. emarginatum* as it was the only species to show quantifiable amounts of ecgonine methyl ester from crude extracts. Ecgonine methyl ester was detected in each season apart from the summer-collected samples. The autumn- and spring-collected samples produced significantly

higher quantities (0.0162 ± 0.0025 % and 0.0160 ± 0.0007 % per gram dry leaf material, respectively) than the summer- and winter-collected samples (0.0000 % and 0.0022 ± 0.0001 % per gram dry leaf material, respectively). The winter-collected samples produced significantly higher quantities as compared to the summer samples at a P-value < 0.05 (R^2 = 0.97). The annual average production of ecgonine methyl ester was 0.0086 % per gram dry leaf material. These findings suggest that accumulation of this compound occurs during autumn and spring, while dissipation thereof seems to occur during summer and winter.

The variation in abundance of plant secondary metabolites as a result of seasonal related biotic and abiotic changes is well established (Treutter 2001; Solar et al. 2006; Taiz and Zeiger 2010; Cramer et al. 2011; Ncube et al. 2015). However, no previous reports could be found describing the variation of cocaine precursor production as a result of seasonal related environmental changes in the *Erythroxylum* species, making this the first report thereof. The results shown above could be the reason for the conflicting reports on the occurrence of tropane alkaloids in the South African species. The authors of these studies do not state in which season the samples were collected and it could have been collected in a season from which there were undetectable quantities of the specific tropane alkaloids, thus emphasising the importance of seasonal variation studies (Evans 1981; Nishiyama et al. 2007). Furthermore, when comparing the average yield of ecgonine methyl ester observed in this study (0.0086 %), to the concentrations found in greenhouse grown, as well as seven-day-old wild grown *E. coca* (0.59 % and 0.24 %, respectively), one could see that the 'old world species' produced vastly lower quantities of this tropane alkaloid (Johnson and Emche 1994; Johnson 1996). This suggests that the environmental selection pressure for this secondary compound and downstream compounds are more severe in the case of *E. coca*.

The phytochemical profiling revealed that most of the analysed classes of secondary compounds were present in all three *Erythroxylum* species, including cardiac glycosides, phenolics, tannins, and

terpenoids. The presence of saponins and alkaloids were only detected in *E. emarginatum* and *E. delagoense*, and not in *E. pictum.* The presence of alkaloids was detected in *E. pictum*, when the more sensitive detection method GC-MS was used, instead of the widely used general phytochemical profiling method of Mushtaq et al. (2014).

The GC-MS screening of alkaloid extracts also displayed the presence (at a ≥ 90 % similarity index) of some highly valued tropane and related alkaloids. Species belonging to the *Erythroxylum* genus are in general well known for their alkaloids, more specifically tropane alkaloids, which are an important group due to their vast range of biological activities (Chin et al. 2006; De Simone et al. 2008; Grynkiewicz and Gadzikowska 2008). The tropane related alkaloids detected and identified in the respective alkaloid screening study can be viewed in Table 1, while Table 2 displays the alkaloids observed when different extraction temperatures were analysed. Representative total ion chromatograms (TIC's) of alkaloid extracts can be seen in Fig. 2. The chemical structure of the identified compounds can be seen in Fig. 3, while Fig. 4 shows the representative head-to-tail mass spectra of each identified compound compared to the NIST reference spectra.

The screening analysis was done on all three species, whereas the PLE temperature analysis was done only on *E. emarginatum*, as it showed during a pilot study, to contain the most diverse groups of tropane alkaloids. A similarity index (SI) or match factor threshold between the observed MS and the NIST 14 library spectra was set at ≥ 90 %. It has been experimentally shown by Stein (1999) that one can be confident in a library to sample MS match above 85 %, given that the correct identification methods are followed. It should be noted that benzoic acid methyl ester was included in the analysis, as it is one of the important precursors present in many highly valued tropane alkaloids (Grynkiewicz and Gadzikowska 2008).

Table 1 Tropane alkaloids observed in the respective *Erythroxylum* alkaloid extracts during the screening analysis

Species	Compound number (Fig. 3 & Fig. 4)	Rt[a] ± SD[b] (minutes)	Compound Name	Quality (SI[c] % ± SD[b])	Molecular Weight (amu)	Presence in number of replicates (n = 5)
E. delagoense	1	3.459 ± 0.00	Benzoic acid methyl ester*	93.0 ± 2.17	136.05	3
	3	4.208 ± 0.12	2-Carbomethoxy-8-methyl-8-azabicyclo[3.2.1]oct-2-ene	96.0 ± 0.49	181.11	5
	4	4.387 ± 0.76	Ecgonidine methyl ester	98.0 ± 0.01	181.11	5
	5	4.455 ± 0.14	Ecgonine methyl ester	94.8 ± 1.17	199.12	5
	6	4.634 ± 0.04	Pseudoecgonine methyl ester	91.0 ± 0.63	199.12	5
E. emarginatum	2	3.789 ± 0.01	Tropinone	90.8 ± 1.30	139.1	4
	3	4.022 ± 0.05	2-Carbomethoxy-8-methyl-8-azabicyclo[3.2.1]oct-2-ene	97.0 ± 0.49	181.11	5
	4	4.376 ± 0.17	Econidine methyl ester	94.4 ± 1.02	181.11	5
	5	4.631 ± 0.02	Ecgonine methyl ester	97.1 ± 0.40	199.12	5
	6	4.746 ± 0.02	Pseudoecgonine methyl ester	90.6 ± 1.20	199.12	5
E. pictum	1	3.456 ± 0.01	Benzoic acid methyl ester*	95.5 ± 1.50	136.05	2
	3	4.025 ± 0.05	2-Carbomethoxy-8-methyl-8-azabicyclo[3.2.1]oct-2-ene	96.5 ± 1.50	181.11	2
	4	4.376 ± 0.02	Ecgonidine methyl ester	97.0 ± 1.00	181.11	2
	5	4.393 ± 0.06	Ecgonine methyl ester	93.7 ± 1.89	199.12	3
	6	4.672 ± 0.05	Pseudoecgonine methyl ester	92.0 ± 1.00	199.12	2

[a]RT – Retention time

[b]SD – Standard deviation

[c]SI – Similarity index

*Important tropane alkaloid precursor

Table 2 Tropane alkaloids observed in *E. emarginatum* alkaloid extracts during the PLE temperature analysis

Extraction temperature	Compound number (Fig. 3 & Fig. 4)	RT[a] ± SD[b] (minutes)	Compound Name	Quality (SI[c] % ± SD[b])	Molecular Weight (amu)	Presence in number of replicates (n = 5)
30 °C	1	3.460 ± 0.00	Benzoic acid methyl ester*	93.2 ± 1.83	136.05	5
	3	4.281 ± 0.12	2-Carbomethoxy-8-methyl-8-azabicyclo[3.2.1]oct-2-ene	97.0 ± 0.00	181.11	5
	4	4.408 ± 0.12	Ecgonidine methyl ester	98.0 ± 0.00	181.11	5
	5	4.535 ± 0.09	Ecgonine methyl ester	93.8 ± 1.64	199.12	4
	6	4.669 ± 0.04	Pseudoecgonine methyl ester	92.7 ± 1.89	199.12	3
	7	6.065 ± 0.04	Tropacocaine/ Benzoyltropeine	93.3 ± 2.31	245.14	5
	8	6.099 ± 0.05	Tropacocaine	95.0 ± 1.00	245.14	4
	9	6.152 ± 0.04	3α -Phenylacetoxytropane	94.8 ± 2.86	259.16	4
40 °C	2	3.789 ± 0.00	Tropinone	94.0 ± 0.00	139.10	1
	3	4.284 ± 0.13	2-Carbomethoxy-8-methyl-8-azabicyclo[3.2.1]oct-2-ene	97.0 ± 0.00	181.11	5
	4	4.420 ± 0.01	Ecgonidine methyl ester	98.0 ± 0.00	181.11	5
	5	4.560 ± 0.05	Ecgonine methyl ester	94.4 ± 2.65	199.12	5
	6	4.685 ± 0.03	Pseudoecgonine methyl ester	91.3 ± 1.10	199.12	4
	8	6.120 ± 0.03	Tropacocaine	95.8 ± 1.92	245.14	4
	9	6.173 ± 0.03	3α -Phenylacetoxytropane	93.2 ± 3.50	259.16	4
60 °C	1	3.469 ± 0.00	Benzoic acid methyl ester*	92.3 ± 0.94	136.05	3
	2	3.811 ± 0.00	Tropinone	93.3 ± 1.17	139.10	1
	3	4.284 ± 0.08	2-Carbomethoxy-8-methyl-8-azabicyclo[3.2.1]oct-2-ene	97.2 ± 0.00	181.11	5

	4	4.421 ± 0.04	Ecgonidine methyl ester	98.0 ± 1.17	181.11	5
	5	4.539 ± 0.12	Ecgonine methyl ester	94.2 ± 1.09	199.12	5
	6	4.679 ± 0.00	Pseudoecgonine methyl ester	91.0 ± 0.71	199.12	4
	7	6.077 ± 0.01	Tropacocaine/ Benzoyltropeine	93.3 ± 3.56	245.14	4
	8	6.111 ± 0.01	Tropacocaine	96.8 ± 1.67	245.14	3
	9	6.167 ± 0.00	3α -Phenylacetoxytropane	94.5 ± 1.41	259.16	4
	10	6.497 ± 0.01	Atropine	91.0 ± 1.41	289.17	3
80 °C	1	3.463 ± 0.00	Benzoic acid methyl ester*	94.5 ± 1.50	136.05	4
	2	3.481 ± 0.00	Tropinone	94.0 ± 0.00	139.10	2
	3	4.278 ± 0.11	2-Carbomethoxy-8-methyl-8-azabicyclo[3.2.1]oct-2-ene	97.0 ± 0.00	181.11	5
	4	4.411 ± 0.01	Ecgonidine methyl ester	98.0 ± 0.00	181.11	5
	5	4.529 ± 0.13	Ecgonine methyl ester	94.6 ± 1.62	199.12	5
	6	4.627 ± 0.04	Pseudoecgonine methyl ester	91.0 ± 1.10	199.12	5
	7	6.065 ± 0.04	Tropacocaine/ Benzoyltropeine	94.0 ± 0.89	245.14	5
	8	6.099 ± 0.04	Tropacocaine	95.2 ± 2.79	245.14	5
	9	6.155 ± 0.04	3α -Phenylacetoxytropane	93.2 ± 2.48	259.16	5
	10	6.497 ± 0.39	Atropine	91.0 ± 1.41	289.17	3

[a]RT – Retention time

[b]SD – Standard deviation

[c]SI – Similarity index

*Important tropane alkaloid precursor

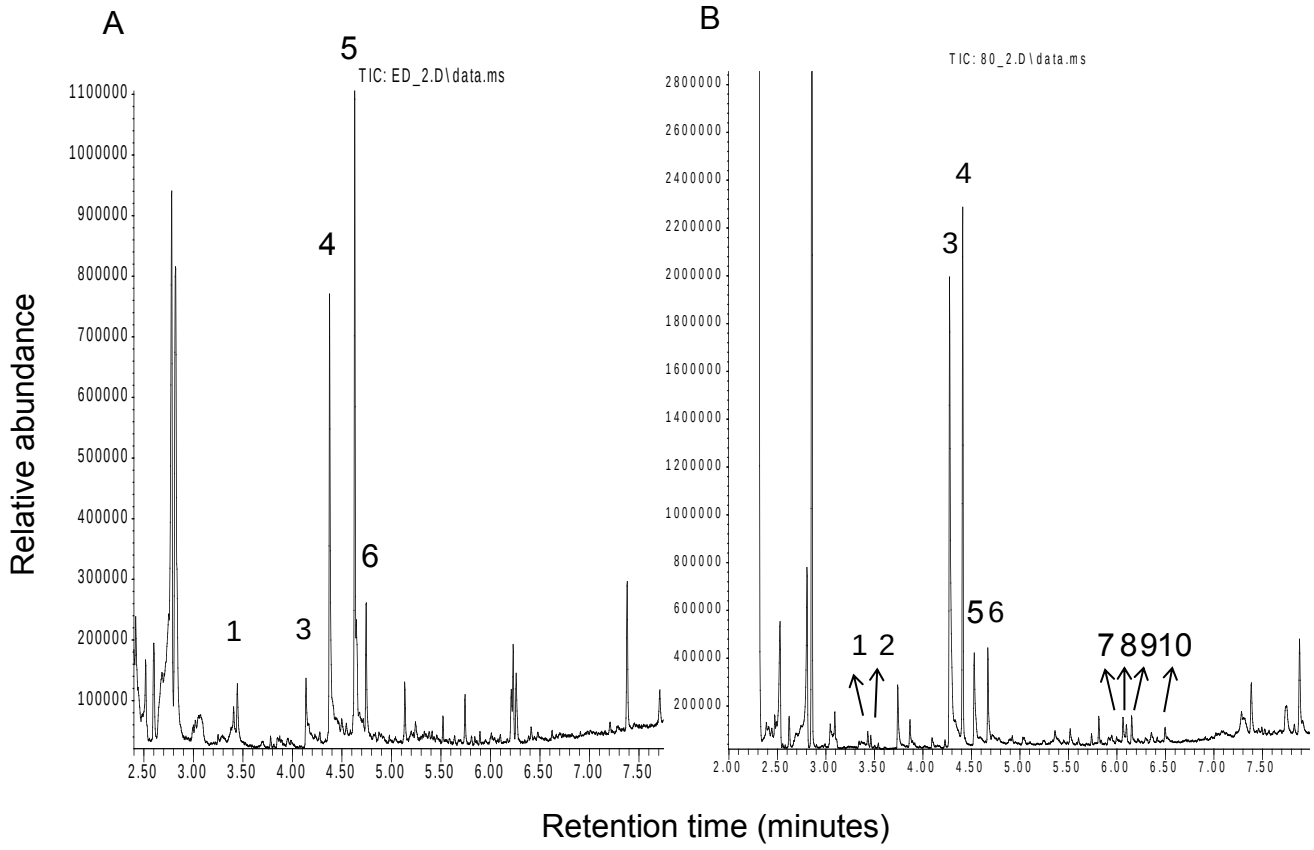

Fig. 2 Representative alkaloid extract total ion chromatograms (TIC's**),** with peaks shown of selected tropane and related alkaloids**.** (A) TIC of *E. delagoense* extracted at 60 °C used in the screening analysis. (B) TIC of *E. emarginatum* extracted at 80 °C used in the PLE analysis. The names and chemical structures of the labelled compounds are depicted in Fig. 3

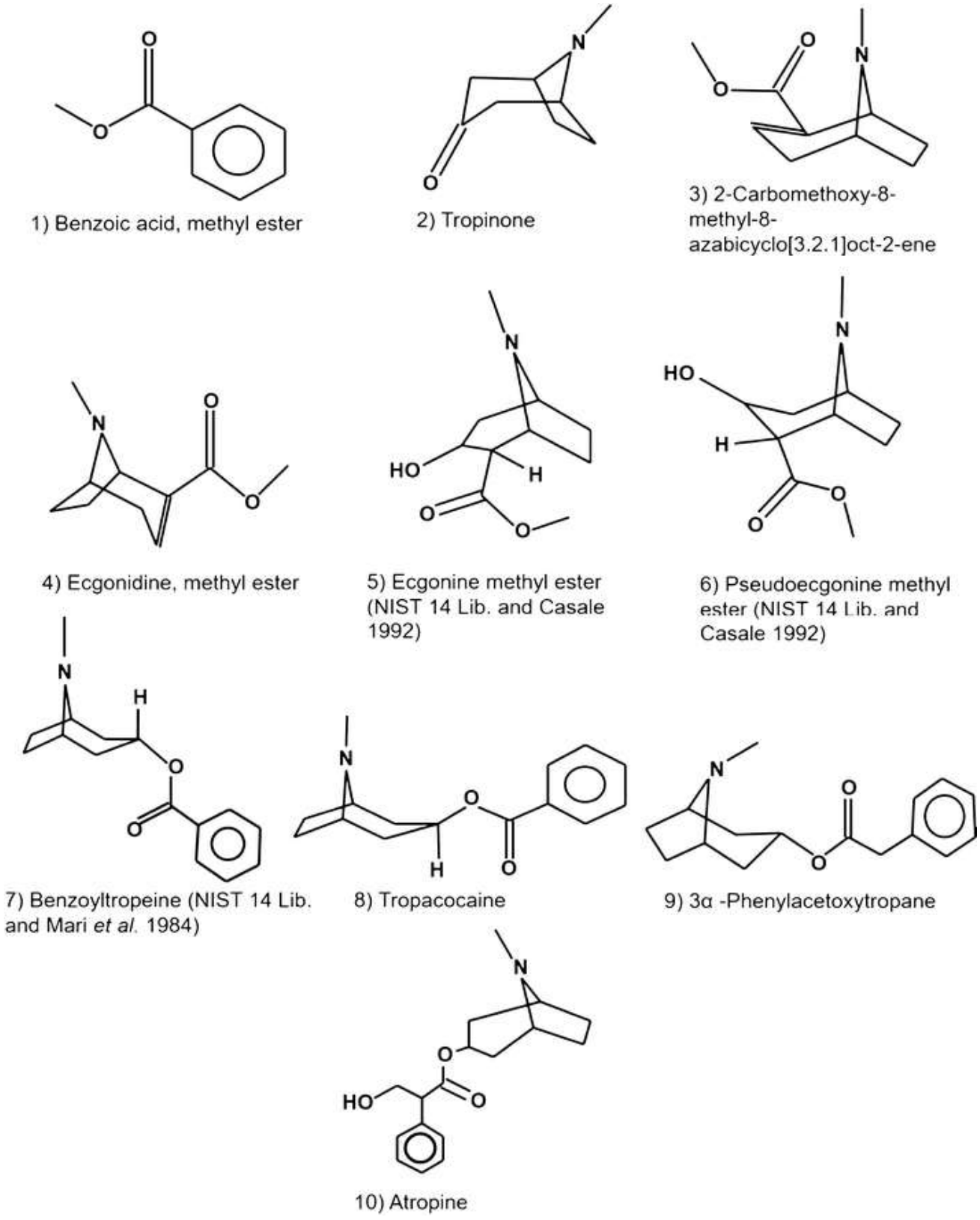

Fig. 3 Chemical structures of the observed tropane and related alkaloids. The chemical structures were obtained from the NIST 14 mass spectral database software, with specific modifications made according to Casale (1990, 1992) and Mari et al. (1984)

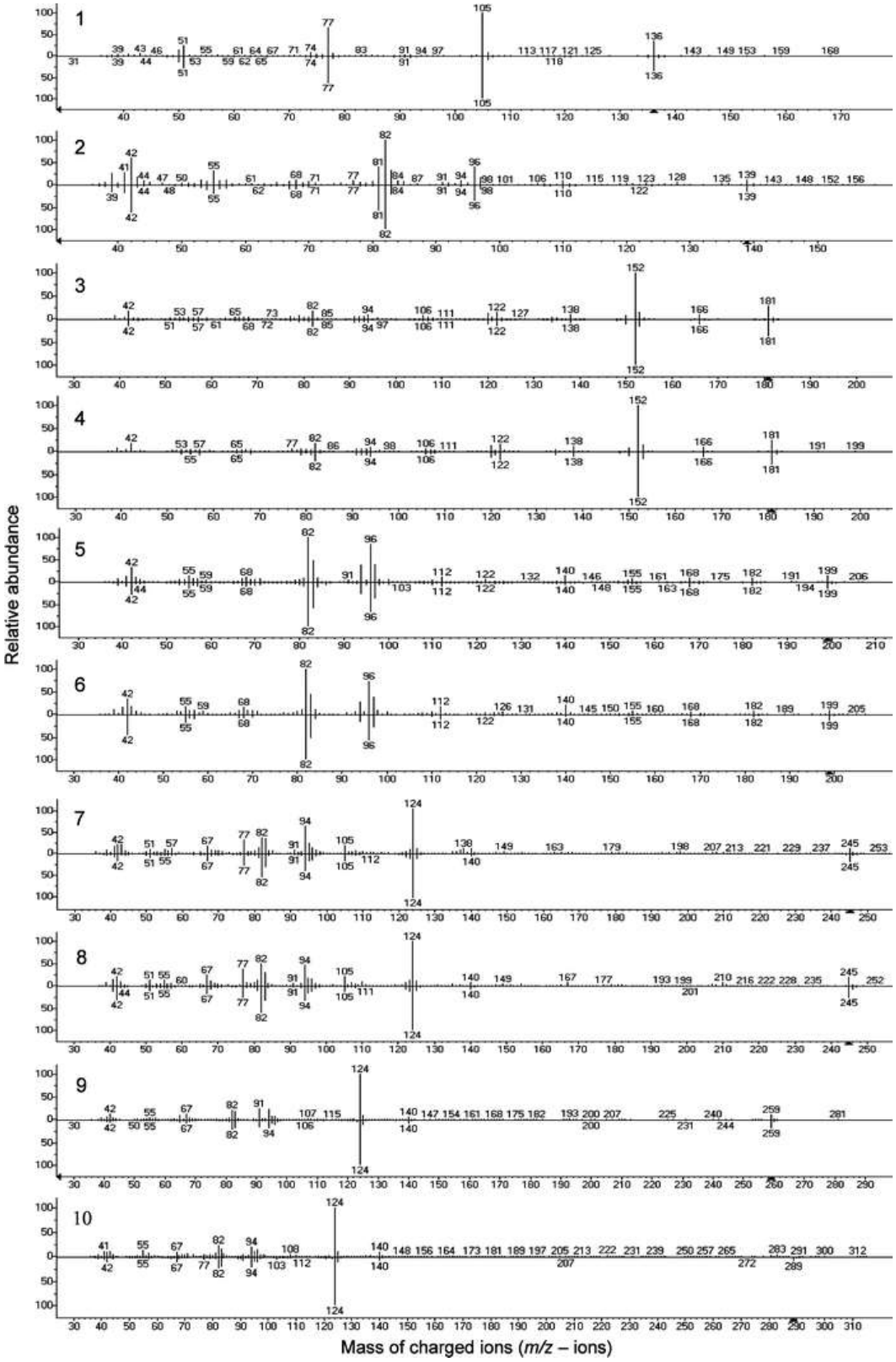

Fig. 4 Mass spectral fragmentation patterns of the identified compounds in the *E. emarginatum* alkaloid extracts (top), compared to the MS of the NIST 14 spectral database (bottom). The names and chemical structures of the labelled compounds are depicted in Fig. 3

The results from Table 1 indicate that 2-carbomethoxy-8-methyl-8-azabicyclo[3.2.1]oct-2-ene, ecgonidine-, ecgonine-, and pseudoecgonine methyl ester were observed in all three species analysed during this study, although 2-carbomethoxy-8-methyl-8-azabicyclo[3.2.1]oct-2-ene, ecgonidine methyl ester, and pseudoecgonine methyl ester is only detected in two of the five replicates of *E. pictum* above a 90 % similarity index. Benzoic acid methyl ester was observed in *E. delagoense* (3/5 replicates) and *E. pictum* (2/5 replicates), while tropinone was only observed in *E. emarginatum* (4/5 replicates).

The results from the PLE temperature analysis (Table 2) revealed that *E. emarginatum*, extracted at all four temperatures, contained 2-carbomethoxy-8-methyl-8-azabicyclo[3.2.1]oct-2-ene, ecgonidine methyl ester, ecgonine methyl ester, pseudoecgonine methyl ester, tropacocaine, and 3α-phenylacetoxytropane. Moreover, tropinone was observed in the extracts at 40 °C (1/5 replicates), 60 °C (1/5 replicates), and 80 °C (2/5 replicates), while benzoic acid methyl ester was observed in the extracts at 30 °C (5/5 replicates), 60 °C (3/5 replicates), and 80 °C (4/5 replicates). The 60 °C and 80 °C samples showed to contain the most diverse groups of tropane alkaloids, including atropine, and those already mentioned above. The 80 °C extraction however, showed to be the most efficient extraction temperature, with a higher number of replicates confirming compound presence. This indicated that a relative high temperature is needed to extract this group of alkaloids when pressurised liquid extractions are preformed, thus correlating with the results obtained by Mroczek et al. (2006) and Brachet et al. (2001).

This is significant in light of the findings by Oliveira et al. (2010) who showed that of the 230 *Erythroxylum* species described in literature, only 35 have been reported to produce tropane alkaloids and of these 10 were able to produce cocaine, 11 produced detectable amounts of ecgonine methyl ester, while only four were found to produce tropinone. It should be noted that, the low number of sample replicates containing a specific compound, could possibly be attributed to interfering baseline

noise because of the detection limit being reached of the apparatus, or co-eluting compounds. These factors would also contribute to not obtaining a ≥ 90% similarity index result, when a NIST search is performed (Stein 1999).

Ecgonidine methyl ester and 2-carbomethoxy-8-methyl-8-azabicyclo[3.2.1]oct-2-ene are stereoisomers of each other, with little known of their stereochemistry (Royal Society of Chemistry 2015a, b). No reports in literature could be found describing their combined presence in plant samples, however the presence of both compounds was observed in the ecgonine methyl ester standard samples used for quantification during the seasonal variation study. Ecgonine methyl ester seemed to elute as a double peak on the GC chromatogram and this can be explained by the presence of the stereoisomer, pseudoecgonine methyl ester (Casale 1992). It has been shown that pseudoecgonine methyl ester elutes as the second peak, while also having a very similar MS fragmentation pattern to that of ecgonine methyl ester (Casale 1990, 1992). This resulted in observing a similar MS match pattern and similarity index values between the two stereoisomers when this peak was analysed (Fig. 4; MS 5 and 6).

The two peaks of tropacocaine seen in the extracts at 30 °C, 60 °C, and 80 °C, may be due to a stereoisomer of tropacocaine being present in the samples. The conformational stereoisomer benzoyltropeine, has been shown by Mari et al. (1984) to have an extremely similar mass fragmentation pattern to that of tropacocaine, although the two compounds showed different GC retention times. The 83 *m/z* – fragment ion is the differentiating factor used to distinguish between the two compounds, with the ion of tropacocaine being of lower intensity, as compared to that of benzoyltropeine (Fig. 5). Benzoyltropeine is not a registered compound on the NIST 14 MS software database, although the compound name is shown as a synonym for tropacocaine. This would have caused the similarity search results to indicate both peaks as being tropacocaine, where the first peaks at Rt – 6.099 (30 °C), 6.077 (60 °C), and 6.065 (80 °C), could rather be the stereoisomer benzoyltropeine based on their MS spectra (Mari et al. 1984).

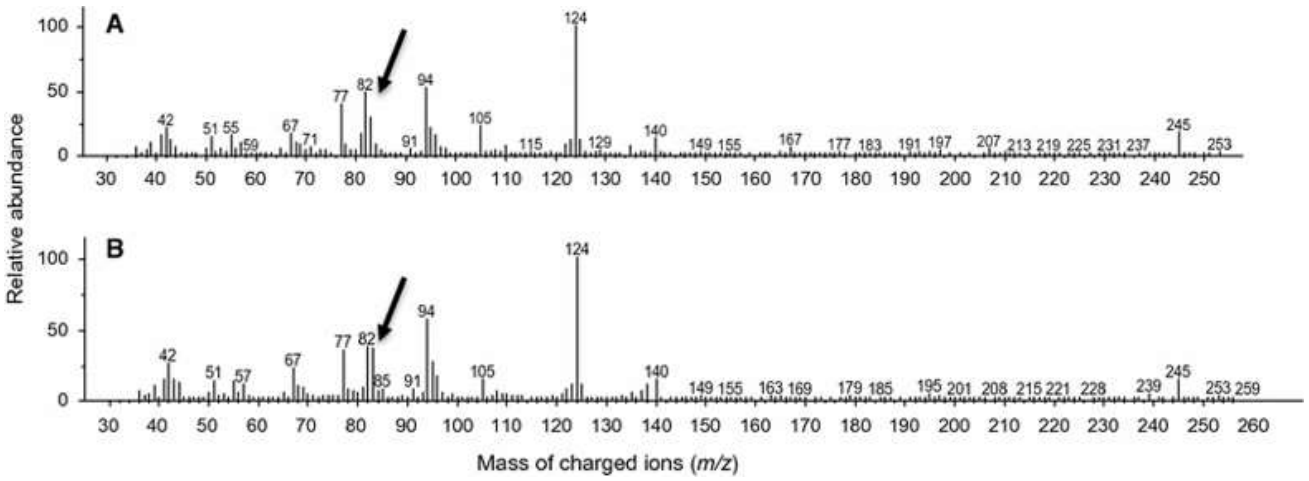

Fig. 5 Tropacocaine and benzoyltropeine mass fragmentation patterns. **(**A) Tropacocaine, identified by the NIST 14 MS software. (B) Possible stereoisomer benzoyltropeine, identified based on the study by Mari et al. (1984). The arrows indicate the ion at *m/z* 83

Conclusion

Based on GC-MS results, tropinone, tropacocaine, 3α–phenylacetoxytropane, and atropine were reported here for the first time to be present in the leaves of *E. emarginatum.* The presence of ecgonidine methyl ester, ecgonine methyl ester, and their stereoisomers were also reported here for the first time to be present in the leaves of *E. delagoense* and *E. pictum*. The optimal extraction temperature for tropane related alkaloids in the South African coca species was 80 °C (of the four temperatures studied). The findings in this study confirmed that tropane alkaloids have a limited distribution within the *Erythroxylum* genus and that the South African *Erythroxylum* species, considered as 'old-world species', contain only selected tropane alkaloids.

The southern African varieties, however, do not contain large quantities of tropane alkaloids and therefore, it can be hypothesised that the reported traditional use of these plants may rather be due to other alkaloids or compound classes found in higher abundance than the tropane alkaloids. Synergy

between the different compound classes, such as flavonoids, phenolics, tannins, and terpenoids, observed in the general phytochemical profiling may also explain the diverse traditional medicinal uses of these species, thus justifying further research on their biological activity.

It would be interesting in future studies to establish whether the cocaine synthase gene is present, mutated or not expressed in the southern African species, as it was established that compounds, such as the immediate cocaine precursor, ecgonine methyl ester, is present in some of the species, but no cocaine was detected in these. Could this indicate a possible obstructed pathway or merely a pathway that has another endpoint than cocaine? Furthermore, would it be possible to increase the production of detected highly valued medicinal tropane alkaloids in tissue culture? Current research is underway in this regard.

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