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Isolation of *Sceletium* alkaloids by high-speed countercurrent chromatography

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ABSTRACT

The alkaloids of *Sceletium tortuosum* (Mesembryanthemaceae) exhibit important pharmacological properties and are used for the treatment of psychiatric and psychological conditions, including depression, anxiety, drug dependence, bulimia and obsessive-compulsive disorder. The all-liquid technique of high-speed countercurrent chromatography provides an excellent tool for the rapid isolation of these alkaloids in high yields.

1. Introduction

Sceletium tortuosum (L.) N.E.Br., also referred to as *Mesembryanthemum tortuosum* L. (Mesembryanthemaceae) is endemic to the Cape Region of South Africa and has probably been used for centuries as a mood-altering drug (Gericke and Viljoen, 2008). It is currently one of South Africa's most important medicinal plants and preparations have, of late, been increasingly sought after. *Sceletium* plantations have been established and diverse consumer products derived from *Sceletium* are being sold commercially.

Alkaloids are known to be the active constituents in *Sceletium* species. Although *S. tortuosum* produces a variety of alkaloids, four of those specifically referred to as mesembrine alkaloids (mesembrine, mesembrenone, mesembrenol and mesembranol) (Fig. 1) have been recognized for their remarkable psychoactive properties (Gericke and Van Wyk, 1997). These compounds are used in pharmaceutical formulations for the management of psychiatric and psychological conditions including depression, anxiety, drug dependence, bulimia and obsessive-compulsive disorder (Gericke and Van Wyk, 1999). The ability of mesembrine alkaloids to treat conditions of the central nervous system (CNS) has been ascribed to their capacity to act as serotonin re-uptake inhibitors, thereby contributing to regulating the balance of neurochemicals in the brain (Gericke and Viljoen, 2008; Patnala and Kanfer, 2009). Convenient methods for the efficient isolation of the alkaloids are urgently required to facilitate further pharmacological studies (Smith et al., 1996), and to develop quality control protocols for plant materials and products.

The total alkaloid yield of *S. tortuosum* is relatively low (0.05–2.3% of dry weight) (Gericke, 2002) and significant yields of compounds can only be realized when large quantities of plant material are used for isolation. Purification methods including column chromatography (CC), preparative high performance liquid chromatography (HPLC) and preparative thin layer chromatography (PTLC) have played a crucial role in the isolation of alkaloids from *Sceletium* plant material (Jeffs et al., 1970, 1982; Snyckers et al., 1971; Gericke and Viljoen, 2008). However, the basic nature of alkaloids is responsible for a number of problems experienced during analysis and purification, including extensive tailing and irreversible adsorption to column packing materials (Yang and Ito, 2005). Therefore, attention was turned to high-speed countercurrent chromatography (HSCCC) to provide more efficient separations. This technique is an all-liquid method, in which separation is achieved by partitioning of the sample components between two immiscible liquids (Ito, 2005). The absence of a solid support allows the entire introduced sample to be recovered. A large number of solvent combinations are possible and many examples of using this technique in the purification of natural products have been documented (Marston and Hostettmann, 2006). Moreover, the technique eliminates adsorption or catalytic changes encountered with solid supports, as well as contamination originating from the leaching of adsorbents.

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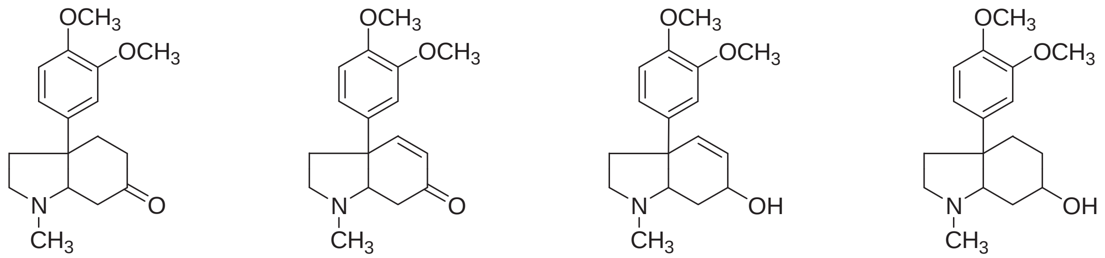

1 (mesembrine) **2** (mesembrenone) **3** (mesembrenol) **4** (mesembranol)

Fig. 1. Structures of principal alkaloids in *Sceletium tortuosum*.

Table 1
Purities and masses of alkaloids isolated from *Sceletium tortuosum* using HSCCC and CC/PTLC techniques.

Compound no.	Compound	Rf value (TLC)	Retention time (GC–MS) (min)	Mass isolated (mg)		Purity (%) (GC–MS)	
				HSCCC	CC/PTLC	HSCCC	CC/PTLC
1	Mesembrine	0.76	18.36	482.4[a]	130.3[c]	98.5	96.0
2	Mesembrenone	0.87	18.82	545.2[a]	40.4[c]	98.4	90.0
3	Mesembrenol	0.38	17.65	300.0[b]	180.6[d]	98.2	87.1
4	Mesembranol	0.27	17.92	47.8[b]	110.1[d]	95.4	80.0

[a] Isolated from 1.5 g Fraction B.
[b] Isolated from 1.0 g Fraction C.
[c] Isolated from 1.75 g Fraction B.
[d] Isolated from 1.65 g Fraction C.

2. Results and discussion

Dilute H_2SO_4 was used to convert the basic alkaloids into salts to enable their initial extraction from the plant material. These salts were then converted back to free bases by the addition of ammonia prior to partitioning with dichloromethane. This technique is commonly used for the isolation of alkaloids from plant material (Katavic, 2005). Analysis of the crude alkaloid extract by TLC indicated the presence of four major constituents with Rf values of 0.87, 0.76, 0.38 and 0.27. The extract was fractionated by silica gel column chromatography with a chloroform–methanol gradient, resulting in four fractions (A–D). Initial work relied on subsequent preparative TLC for purification of the four alkaloids, but this was a tedious method, which gave low yields (Table 1).

The purities of compounds obtained following CC separation were compromised by overlap of the components during elution from the columns. This observed tailing was the result of the interaction of the basic alkaloids with the acidic silanol groups of the silica gel packing material (Liu et al., 2009). Further separation was conducted by applying fractions containing the compounds of interest to successive columns, until the number of compounds in the fractions was sufficiently reduced to enable separation by preparative TLC (PTLC). However, the purity of the alkaloids obtained by scraping the bands from PTLC also proved to be unsatisfactory due to overlap of bands caused by tailing.

Preparative TLC analysis is a costly process, particularly when compounds are required in significant quantities (>100 mg). Many plates are required in order to obtain good yields. In this study, the amount of alkaloid extract that could be applied to each TLC plate was limited to ~100 μg, while the relative amount of each alkaloid obtained per plate was also very small (<50.0 μg).

Separation of mesembrine (**1**) and mesembrenone (**2**) by HSCCC was achieved directly from a crude sample (Fig. 2). The purities of each of the isolated compounds were confirmed by HPTLC and GC–MS. This latter technique was also used to determine the molecular masses of **1**–**4**. The structures of **1**–**4** were confirmed from the MS data and from NMR data (Jeffs et al., 1970).

Higher yields of pure compounds were obtained from smaller quantities of extracts when countercurrent chromatography was used for separation instead of the CC-PTLC combination. During HSCCC isolation, only 1.5 g of the 3.25 g of Fraction B was used to produce 482.4 mg and 545.2 mg of compounds **1** and **2**, respectively (Table 1). For Fraction C, 1.0 g from 2.65 g was used to obtain 300.0 mg and 47.8 mg of compounds **3** and **4**, respectively (Table 1). These results were in contrast to those obtained by CC, where 1.75 g of Fraction B and 1.65 g of Fraction C were separately applied to additional columns for purification. The final fractions from CC were further purified using PTLC. The

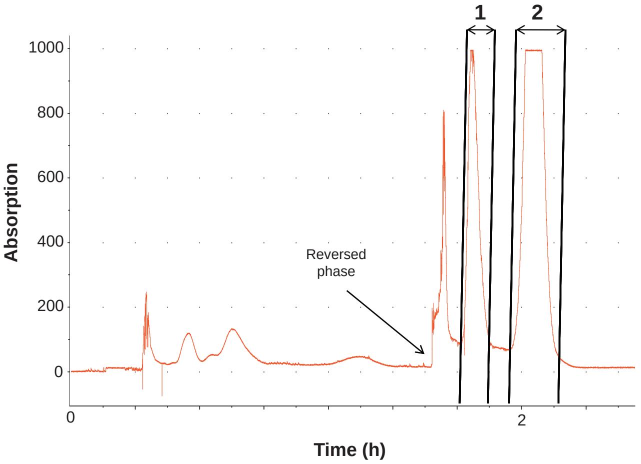

Fig. 2. Chromatogram (HSCCC) of Fraction B from silica gel column chromatography. Solvent: n-heptane–MeOH–EtOAc–1% NH_3 (1:3:1:3, v/v) (mobile phase = upper phase to 100 min). Sample: 1.5 g of Fraction B was dissolved in a mixture of the upper (2.0 ml) and the lower phases (2.0 ml) and filtered, prior to injection into the instrument. Operating temperature: 30 °C. Coil rotation speed 1500 rpm. Flow-rate 3.0 ml/min. **1**: mesembrine, **2**: mesembrenone.

masses of three of the compounds obtained (**1**: 130.3 mg, **2**: 40.4 mg and **3**: 180.6 mg) using these techniques were substantially lower than those obtained from HSCCC separation. However, the mass of compound **4** from CC-PTLC (110.1 mg) separation was more than that from HSCCC (47.8 mg), although the purity was lower (80.0%). The additional clean-up steps required to remove the contaminants would, however, have resulted in a lower final mass of pure compound. When a CC-PTLC combination is used for isolation, the low recoveries of compounds may be attributed to losses arising from irreversible adsorption to the silica gel phase (Conway, 1990) and to the small amounts of compounds discarded after each column, during pooling of fractions, to conserve the purity of the combined fractions. These considerations also limit the use of the two techniques for isolation of the alkaloids, which are reported to occur in organisms in small quantities. Since the total alkaloid yield of *S. tortuosum* was quite low (1.94% of dry weight), large amounts of plant material would be required to obtain significant quantities of pure compounds when both CC and PTLC are used for isolation.

The problem is eliminated with the use of HSCCC since all the constituents in the sample can be recovered by evaporation of both liquid mobile and stationary phases (Conway, 1990). HSCCC was found to be cost effective, since the time required for isolation and the solvent consumption were much lower than those required for CC. A total volume of 400 ml of solvent mixture was sufficient for a complete one-step HSCC separation of compounds from one fraction in only 3 h.

3. Experimental

3.1. General experimental procedures

The hydrodynamic HSCCC instrument used in the present study was a Spectrum model (Dynamic Extractions, Slough, UK) multilayer coil-planet J-type centrifuge, equipped with two preparative coils connected in series (wrapped with polytetrafluoroethylene PTFE tubing, 1.6 mm i.d., 142 ml total volume). The inner β_r-value was measured to be 0.52 at the internal end of the coil and outer β_r-value was 0.86 ($\beta_r = r/R$, where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder axis and central axis of the centrifuge). The direction of rotation determined the *head* locations at the periphery of the two coils. Solvent was pumped with a model 305 pump (Gilson, Middleton, WI, USA) and monitoring of the effluent was achieved with a model 151 variable wavelength detector (Gilson, Middleton, WI, USA) at 254 nm. Fractions were collected with a model FC204 fraction collector (Gilson, Middleton, WI, USA). A manual sample injection valve with a 5.0-ml loop from Rheodyne (Rohnert Park, CA, USA) was used to introduce the sample into the column.

NMR spectra were recorded on a Bruker 600 Avance II NMR at 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR. 2D NMR experiments were performed using standard Bruker microprograms. Measurements were made in $CDCl_3$, using solvent signals for calibration.

For GC–MS, samples were analysed with an Agilent 6890N GC coupled to a 5973 MS. Splitless injection (2 μl) was achieved at 255 °C with an auto-injector and an auto sampler at 12.54 psi. The GC system was equipped with a HP-5MS 5% phenyl methyl siloxane column (30 m × 250 μm i.d. × 0.25 μm film thickness). The oven temperature program was initially held at 60 °C, rising to 255 °C at a rate of 20 °C/min and held for 15 min. Helium was used as the carrier gas at a flow rate of 0.7 ml/min and spectra were obtained following electron impact at 70 eV (35–550 *m/z*). Compound purity was calculated from the integrated peak area.

TLC analysis was carried out on Alugram Sil G (UV_{254} aluminium-backed plates, using the solvent $CHCl_3$–CH_3OH-10% NH_3 (90:10:0.1) and Dragendorff reagent for detection (Wagner and Bladt, 2001).

All organic solvents used for extraction and isolation were obtained from Merck Chemical Co., South Africa and were analytical reagent (AR) grade.

3.2. Plant material

Aerial parts of cultivated *S. tortuosum* were obtained from the Western Cape South Africa in April 2009. A voucher specimen (SC 626) is kept at the Department of Pharmaceutical Sciences, Tshwane University of Technology.

3.3. Extraction

Ground, air-dried aerial parts (500 g) were extracted with 0.25 M sulfuric acid (3 × 6 L). The vegetable material was shaken with acid for 15 min with each batch of acid and then left to settle for 10 min, before filtration. Combined filtrates were made alkaline with 20% aqueous ammonia solution (750 ml) and the alkaloids were extracted with dichloromethane (3 × 875 ml). The dichloromethane extract was evaporated to dryness, to yield 9.68 g extract (1.94%).

3.4. Isolation of alkaloids by silica gel column chromatography and preparative TLC

The alkaloid extract (9.68 g), dissolved in $CHCl_3$, was fractionated by silica gel column chromatography (CC) on a glass column (7.0. cm i.d. × 75 cm) packed with 200 g Kieselgel 60 (Macherey-Nagel, Germany). A $CHCl_3$–MeOH gradient (100:0, 99.5:0.5, 99:1, 97:3, v/v) was used for elution of the compounds of interest from the column. Fractions obtained from the column were analysed by TLC using $CHCl_3$–MeOH–10% NH_3 (90:10:0.1, v/v) as developing solvent and subsequently combined to form Fractions A, B, C and D with masses 0.64 g, 3.25 g, 2.65 g and 0.82 g, respectively. Fraction B consisted primarily of compounds **1** and **2**, while Fraction C contained a mixture of compounds **3** and **4**, which were the target alkaloids. Fractions A and D contained compounds which did not react with Dragendorff reagent and hence their separation was not pursued.

3.5. HSCCC fractionation

High-speed countercurrent chromatography was used to separate compounds **1** and **2** from silica gel CC Fraction B and compounds **3** and **4** from Fraction C. A suitable solvent combination (n-heptane–MeOH–EtOac–1% NH_3 (1:3:1:3, v/v) was selected for HSCCC separation of the compounds, after evaluating a variety of solvent mixtures using the method described by Marston and Hostettmann (2006). A portion (0.6 g) of Fraction B was dissolved in a mixture of the upper (2.0 ml) and the lower phases (2.0 ml) and filtered, prior to injection into the chromatograph, which was operated in the normal mode at 30 °C. The coils, without rotation, were first filled with stationary phase. Then rotation of the coils (1500 rpm) was started and mobile phase (upper phase) was pumped at 3.0 ml/min (130 psi) until mobile phase alone exited the coils. It was at this point that the sample was introduced *via* the sample loop. The instrument was run in the normal phase mode for about 100 min before reversing the flow direction and beginning elution with the lower phase (Fig. 1). Alkaloids **1** (mesembrine) and **2** (mesembrenone) were then eluted with baseline separation.

Countercurrent chromatographic separation of Fraction C was also conducted using the same solvent combination and conditions to yield compounds **3** and **4**.

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