Journal of Ethnopharmacology 121 (2009) 86–91

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Investigations of the phytochemical content of *Sceletium tortuosum* following the preparation of "*Kougoed*" by fermentation of plant material

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ARTICLE INFO

Article history:
Received 5 May 2008
Received in revised form 5 August 2008
Accepted 5 October 2008
Available online 17 October 2008

Keywords:
Sceletium tortuosum
Fermentation
Mesembrine
CAMs
Kougoed

ABSTRACT

Aim of the study: Sceletium plant species that contain alkaloids are claimed to have mood elevation and anti-anxiety properties, especially after the plant material has been fermented. The fermented preparation is locally known as "*kougoed*" or "*channa*" and has been emphasized and advertised for its increased potency when incorporated in commercial products. The aim of the study was to investigate quantitative and qualitative changes in alkaloidal content following fermentation of plant samples carried out under controlled conditions and also on pure mesembrine hydrochloride (MHCl).
Materials and methods: Samples were prepared from the aerial parts of *Sceletium tortuosum*. Studies were also conducted on mesembrine hydrochloride (MHCl) in aqueous and methanolic solutions under similar conditions of exposure to sunlight as well as under ambient and elevated temperature ($40 \pm 2\,^{\circ}C$). Quantitative and qualitative changes in alkaloidal content were monitored by HPLC and LC–MS, respectively.
Results and Conclusions: The initial fermentation study showed transformation of mesembrine to Δ^7mesembrenone, where the content of the former decreased from a concentration of 1.33% to 0.05% whilst the latter increased from below its limit of quantitation (LoQ) to 0.11% on the 10th day. The experiments on pure MHCl revealed similar transformations in aqueous solutions whereas no change was seen in methanolic solutions. Sunlight and aqueous conditions appear necessary to facilitate the transformation, which was confirmed by the absence of such a transformation when solutions of MHCl were kept in the dark.

1. Introduction

Phytochemical constituents in plants are reported to vary due to differences in growth conditions, mode and time of harvesting as well as drying and storing the harvested material (Sombra et al., 2005) and there are studies that have shown variations in the phytochemical content between plants grown in a field and greenhouse (Coucerio et al., 2006). The geographical influence on chemical components in cultivated plants has also been reported (Smith et al., 1996). It has been reported that whereas *Sceletium* species cultivated in Germany did not contain alkaloids, those cultivated in the USA did (Herre, 1971). Such variations have important implications for the content and consistency of phyto-pharmaceutical products. In some cases, where plants are reported to be processed in a specific manner, for example, fermentation before production of dosage forms, may result in further variations.

The genus *Sceletium* (family Aizoaceae) occurs in the Western, Eastern and Northern Cape Provinces of South Africa. *Sceletium* occurs more prominently in the Little, Great and Upper Karoo regions. It is also reported to occur in the Namaqualand Rocky Hills, Knersvlakte and Ceres Karoo (Gerbaulet, 1996). The traditional preparation of this plant has been reported as "*Kougoed*" or "*Channa*", which is a fermented preparation used by the native Bushmen of Namaqualand. *Sceletium* is mainly used for its psychoactive properties by the Khoisan tribe of southern Africa and the traditional preparation made by a fermentation process, is purported to enhance the psychoactive effect of the plant (Smith et al., 1996, 1998). *Sceletium* plants and their products are being marketed with claimed improvements of mood and reduction of anxiety, especially when the fermented plant material is either chewed or smoked. Currently there are many websites on the internet that discuss the process of fermentation and make claims that products subjected to a fermentation process result in enhanced pharmacological effects The United States patent on *Sceletium* (Gericke and Van Wyk, 2001) has claimed the pharmacological activity of mesembrine and related compounds in their patent text as an invention. These compounds are mentioned to be useful as serotonin-uptake inhibitors in treatment of mild to moderate

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doi:10.1016/j.jep.2008.10.008

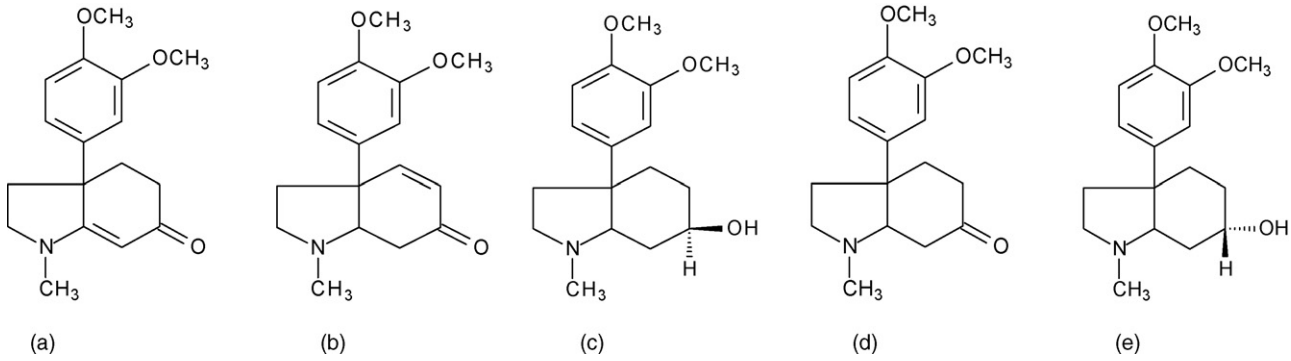

Fig. 1. (a) Δ^7 mesembrenone, (b) Δ^4 mesembrenone (also known as mesembrenone), (c) mesembranol, (d) mesembrine and (e) epimesembranol.

depression, physiological and psychiatric disorders such as anxiety, major depression, and bulimia nervosa and for obsessive compulsive disorders.

The fermentation process in which whole plant material or arial parts are crushed and then placed in sealed containers for several days and dried under natural sunlight is commonly used. Fermentation has been highlighted as one of the important treatments of *Sceletium* plant material (Smith et al., 1996) and this process has been used to advertise increased potency of such products which are marketed as fermented and dried plant powder. The powder is further used in the preparation of commercial phytopharmaceutical dosage forms containing *Sceletium.*

Smith et al. (1996) detailed the fermentation technique based on information obtained from a traditional user of *Sceletium*. The process involved crushing the plant material using stones and allowing the crushed plant material to remain in a bag for about 2–3 days after which the bag was opened, mixed and closed again. Fermentation was then continued for 8 days and the contents were removed from the bag and spread out to dry in the sun. It was emphasized that if the described process was not followed, the product would not be effective for its indicated mood elevating effects.

Smith et al. (1998) reported that the alkaloidal constituents in the genus, *Sceletium,* were of higher concentration only after fermentation. *Kougoed*, when prepared by the contemporary plastic bag method has been reported to contain the mesembrine alkaloids at levels and ratios substantially different from those of unfermented material. When *Sceletium* plant material was dried after harvesting and screened for the presence of alkaloids, the uncrushed dried material revealed the presence of 4′-*O*-demethylmesembrenol, mesembrine and "unspecified" mesembrenone. Although these authors stated that "substantial increases in total alkaloid levels were observed when the *Sceletium* material was crushed and bruised prior to drying, for alkaloid extraction whereas no such changes occurred when intact plants were oven dried at 80 °C prior to alkaloid extraction", no additional comparative data were provided to confirm this statement. However, when fresh plant material of *Sceletium* was crushed and immediately dried at 80 °C, the chromatographic profile was observed to be similar to that of a fermented sample, showing a completely diminished presence of 4′-*O*-demethylmesembrenol, an approximately 50% reduction in mesembrine content and doubling of the "unspecified" mesembrenone peak (Smith et al., 1998). This therefore indicates that the ratio of alkaloidal components was altered rather than there being substantial increases in "total alkaloid levels".

It has been suggested that an enzymatic reaction may occur during the process of bruising the plants and that these reactions may explain the changes in alkaloid ratio and content. Also, the temperature (80 °C) probably influences the changes in alkaloidal content. A second experiment (Smith et al., 1998) was carried out by suspending the crushed plants in liquid nitrogen in order to assess the enzymatic influence. When the frozen plant material was re-suspended in water, the enzymatic activity resumed but was eliminated by boiling in ethanol. The authors thus suggested that the essential step in the production of *Kougoed* may not be entirely due to "fermentation" but that crushing the plant material and consequent mixing of cellular material may also be equally necessary. Based on these results, it was suggested that instead of performing a "traditional" fermentation, simply crushing and drying at 80 °C may be a quick alternative method to modify the alkaloid content. It appears that such treatments may have a rational pharmacological basis which is considered to have evolved over many generations by continued experimentation by indigenous people of southern Africa (Smith et al., 1998).

The content of the major alkaloid, (−)-mesembrine (Fig. 1d), is reported to be approximately 1% in *Sceletium namaquense* and known to occur as a partial racemate in *Sceletium strictum* whereas it is known to occur in smaller amounts in *Sceletium tortuosum* (Jeffs, 1981). Other alkaloids, such as Δ^7mesembrenone, Δ^4mesembrenone, mesembranol and epimesembranol (Fig. 1a–c and e) are also present in the plant and were generally found to be present in lower quantities than mesembrine (Patnala, 2007).

2. Methodology

2.1. Reagents and materials

HPLC grade methanol (UV cutoff 215 nm) and acetonitrile (UV cutoff 200 nm) (Romil Ltd., Cambridge, Great Britain) and ammonium hydroxide 25% solution (Associated Chemical Enterprises (Pty) Ltd., Southdale, South Africa) were used for sample preparation and for the mobile phase. Water was purified in a Milli-Q® system and Millex HV® hydrophilic PVDF 0.45 μm membrane filters (Millipore, Bedford, USA) were used during sample preparation. *Sceletium tortuosum*, was collected from a greenhouse in Robertson, South Africa. The specimens, following identification, were deposited at the Selmar Schonland Herbarium (GRA), Grahamstown, South Africa (Specimen number SP04). The reference compounds, mesembrine, mesembrenone and Δ^7mesembrenone were isolated from *Sceletium* plant material. The compounds mesembranol and epimesembranol were synthesized by catalytic hydrogenation of mesembrine. These compounds were qualified as reference substances by NMR analysis (^{1}H, ^{13}C and 2-D). Mesembranol and mesembrine hydrochloride (MHCl) were obtained in crystalline form and were further characterized by X-ray crystallography (Patnala, 2007).

2.2. Instrumentation

An Alliance 2690 HPLC connected to a photodiode array (PDA) detector 2996 (Waters Corporation, Milford, MA, USA) was used for quantitative analysis and separation of alkaloids was carried out on a Luna® C_{18} (2), 5 μm, 150 mm × 4.6 mm i.d. (Phenomenex®, Torrence, CA, USA) column. An analytical balance, Type AG 135 (Mettler Toledo, Switzerland) was used for weighing standards and samples. An electronic pipette (model 71050XET, Biohit PLC, Helsinki, Finland) was used to transfer standard and sample solutions for dilutions. A Finnigan MAT LCQ ion trap mass spectrometer (Finnigan, San Jose, CA, USA) coupled to a SpectraSYSTEM P2000 pump connected to an AS1000 auto sampler and UV1000 variable-wavelength UV detector (Thermo Separation Products, Riviera Beach, FL, USA) was used for both qualitative and quantitative analysis. A hot air oven Model FSIE and a low temperature incubator, Model L.T.I.E (Labcon (Pty) Limited, Krugersdorp, South Africa) were used to dry the samples and for the temperature controlled studies, respectively. NMR analysis was performed on a Bruker Advance DRX 400 MHz NMR spectrometer (Rheinstetten, Germany).

2.3. Preparation of standard solutions

Standard methanolic stock solutions (1 mg/ml) of Δ^7mesembrenone, mesembranol, mesembrenone, MHCl and epimesembranol were prepared. Calibration standards were prepared to obtain 10 calibrators in the concentration range of 0.4–20 μg/ml using methanol (Fig. 2).

2.4. Preparation of Kougoed

Two separate fermentation studies on *Sceletium* plant material were conducted. The first study involved the use of samples made from the arial plant parts of *Sceletium tortuosum* (75 g) which were transferred into a polythene bag and carefully crushed by hand using fingers, which yielded a watery plant mass. The second fermentation investigation, conducted 12 months after the first study, was carried out in a similar manner but using ~130 g of the same plant's arial parts. The study periods were chosen to coincide with the summer season and hot days, since the natural habitat of *Sceletium* is in the hot and arid Karoo regions of South Africa.

A validated HPLC method was used for the quantitative determination of the relevant alkaloids, which showed limits of detection (LoDs) and limits of quantitation (LoQs) of each of the five alkaloid standards at 100 and 200 ng/ml respectively using the respective S/N ratios of 3 and 10. The relative standard deviation (R.S.D.) for inter-day precision was found to be less than 3.4 and 1.7% and the intra-day precision was found to be less than 8.8 and 7.6% for Δ^7mesembrenone and mesembrine, respectively (Patnala, 2007). The HPLC analyses were performed on the wet mass on day 1 immediately after crushing and the remaining material was left to ferment under sunlight during the day and remained in place throughout the night. Subsequent samples were removed and analyzed each day (24 h intervals) for a period of 10 days for the first fermentation study whereas sampling was extended for 14 days for the second fermentation study. Samples from the second fermentation study were analyzed by LC–UV-MS for quantitative analysis and peak identification.

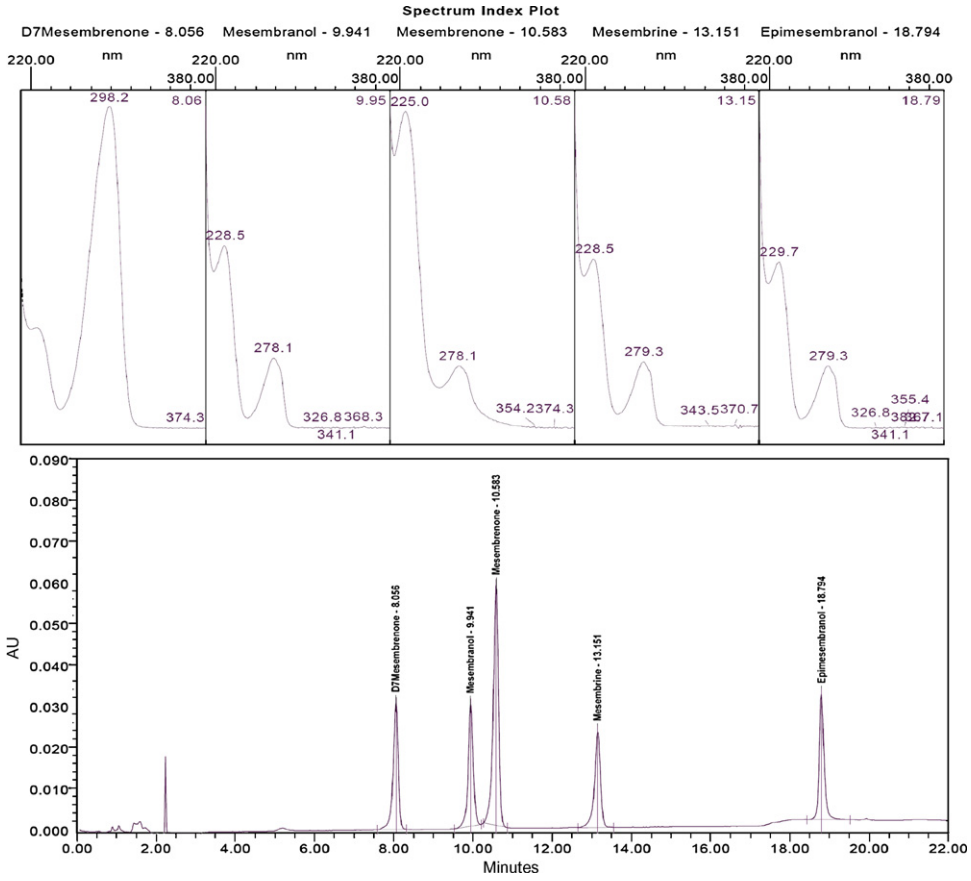

Fig. 2. Sceletium standards. PDA-UV scan of the relevant alkaloids (top) and HPLC chromatogram (bottom).

One plant sample was separately crushed and dried at 80 °C for 5 h according to the method described by Smith et al. (1998), to investigate whether that drying process would provide similar results showing an increase in mesembrenone content.

2.5. Preparation of sample solutions

Three to 5 g of crushed plant material was extracted twice using two portions of 20 ml methanol. These were combined and made up to 50 ml with methanol. The solution was filtered and 1.0 ml was diluted to 10 ml and an aliquot of 10 μl was injected into the HPLC.

2.6. Preparation of mesembrine hydrochloride solutions

An additional study was designed to investigate the transformation of the alkaloids, mesembrine and Δ^7mesembrenone. Standard MHCl was prepared in water to obtain a solution of 0.5 mg/ml. The sample was exposed to the same sunlight exposure conditions as the fermentation studies performed on the plant samples. The solution was sampled on each day at the same times of the plant sample preparation and analyzed under the same conditions.

A further study was designed to investigate the effect of light and temperature on different solutions of MHCl, methanol and water, respectively. The samples were divided into two sets which were protected from light. One set was maintained at 40 °C in a low temperature incubator and the other at ambient temperature (~22 °C).

3. Results

3.1. Sceletium plant fermentation studies

The fermentation studies involving *Sceletium* plant material showed interesting transformations of two prominent alkaloids, mesembrine and Δ^7mesembrenone. The amount of the former decreased whilst the latter increased. A previous study by Smith et al. (1998) reported the transformation of mesembrine and a non-specified mesembrenone and showed similar trends.

The sample on day 1, analyzed immediately after crushing, showed a concentration of 1.33% mesembrine and the presence of Δ^7mesembrenone which was confirmed by PDA analysis, albeit at very low detection levels (<LoQ). When an aliquot of the same crushed sample was dried at 80 °C as performed by Smith et al. (1998), no significant change in mesembrine (1.12%) or in the Δ^7 mesembrenone content (still below the LoQ) was observed. This was in contrast to the results reported by Smith et al. (1998), who found high concentrations of mesembrenone following the same drying procedure. The sample on day 5 showed concentrations of Δ^7mesembrenone, now >LoQ, of 0.07% with the mesembrine content having decreased to 0.68%.

During the course of the fermentation study, the mesembrine content showed a steady decline from an initial 1.33 to 0.05% on the 10th day. On the other hand, the content of Δ^7mesembrenone was found to increase from below the LoQ on days 1–4 to 0.11% on the 10th day. The samples were monitored by an LCMS method (Patnala, 2007) to identify the alkaloids based on their specific *m*/*z* masses as $[M+H]^+$ ion for mesembrine *m*/*z* 290.2 and Δ^7mesembrenone *m*/*z* 288.2 (Figs. 3 and 4).

A graphical representation of the mesembrine and Δ^7mesembrenone content during the first fermentation process is shown in (Fig. 5). It was also observed that no significant change in content of mesembranol, mesembrenone and epimesembranol occurred during the entire fermentation process (content of mesembranol, mesembrenone and epimesembranol were found to be reasonably constant at ~0.14, ~0.15 and ~0.4%, respectively).

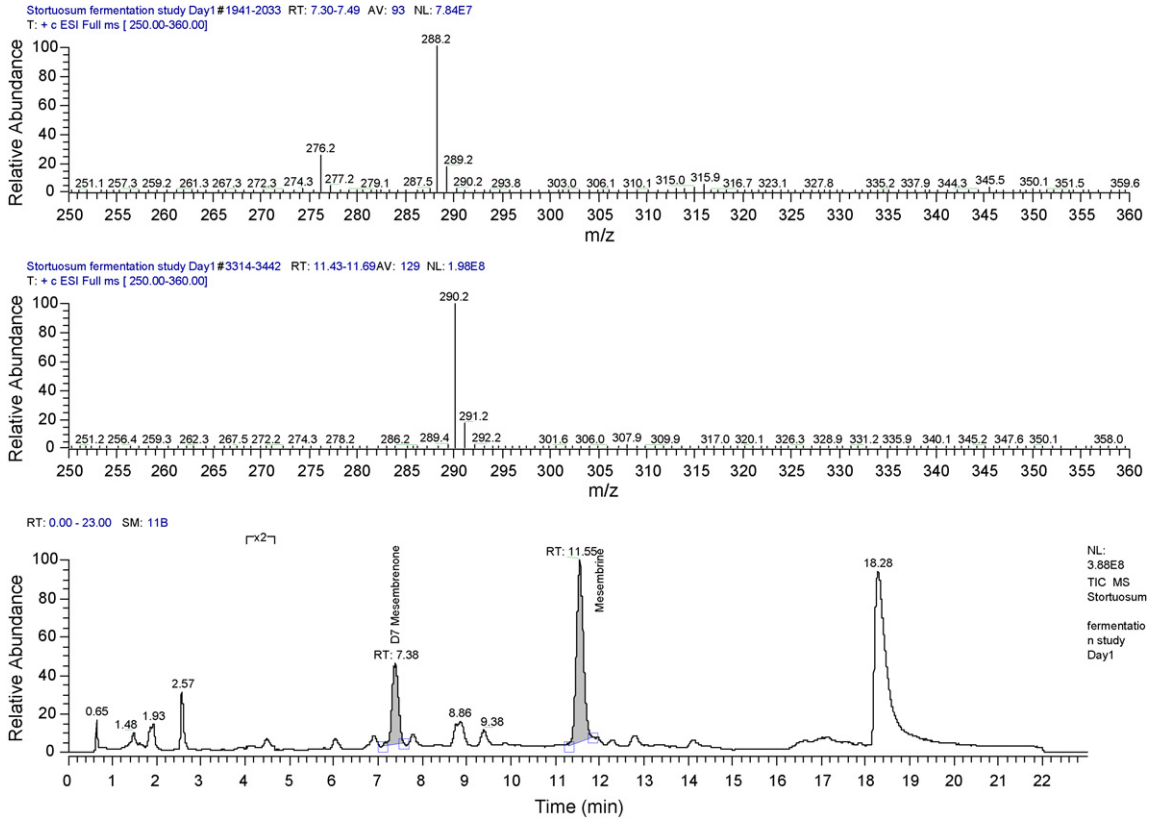

Fig. 3. LC–MS chromatogram of initial crushed plant material on day 1. Ion spectra of Δ^7 mesembrenone *m*/*z* 288. 2 (top), mesembrine *m*/*z* 290.2, (middle) and TIC (bottom).

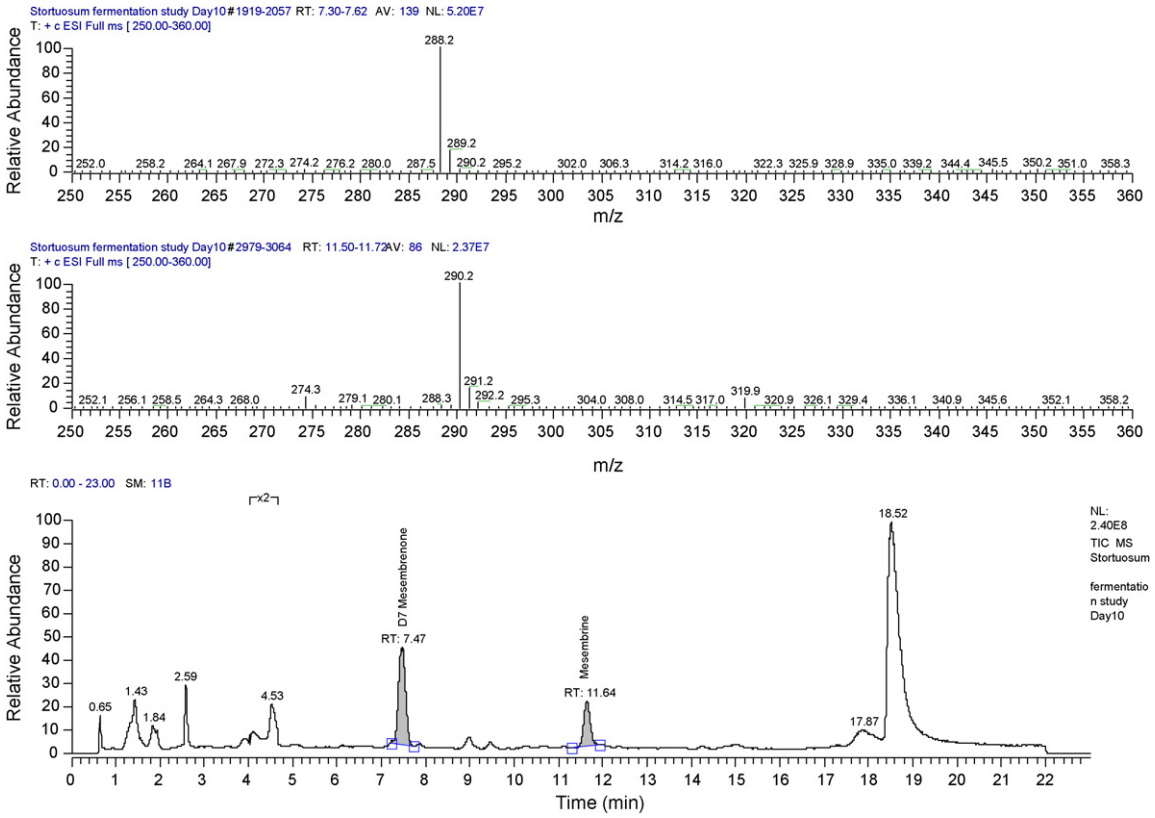

Fig. 4. LC–MS chromatogram of crushed plant material on day 10. Ion spectra of Δ^7 mesembrenone *m*/*z* 288. 2 (top), mesembrine *m*/*z* 290.2 (middle) and TIC (bottom).

The second fermentation study (carried out using the same plant but 1 year later following further growth of that plant) was carried out for 14 days and also showed a decrease in mesembrine content with a concurrent increase in Δ^7mesembrenone. However, the transformations were slower compared to the first fermentation study. The initial mesembrine content for the day 1 sample was found to be 2.2% with mesembrine content decreasing to 0.8% by day 14. Whilst the Δ^7mesembrenone content was found to be below the LoQ from days 1–5, a value above the LoQ of 0.06% was subsequently determined and which increased to 0.18% on day 14.

3.2. Mesembrine hydrochloride studies

The results of this study showed transformation of mesembrine and Δ^7mesembrine in aqueous and in methanolic solutions under similar conditions of exposure to sunlight as during the fermentation studies. The LC–MS analysis of the MHCl in water showed a gradual transformation to Δ^7mesembrenone over a period of 14 days. On day 14, only 35% mesembrine remained whereas 65% Δ^7mesembrenone was now found in the aqueous solution. Interestingly, no alkaloids were found in the same solution when tested after 20 days. In contrast, the sample in methanol showed no such transformation and unlike the aqueous solutions, was unaffected by light. It thus seems that the alkaloid appears to be stabilized in the presence of methanol.

In order to confirm the influence of light and any temperature effects, a study was conducted on samples in water under light protected conditions carried out at room temperature ($22 \pm 2\,^{\circ}C$) and at $40\,^{\circ}C$ after 5 days where no transformation of mesembrine was observed. This study thus confirmed that light affects the stability of mesembrine resulting in its transformation to Δ^7mesembrenone in aqueous solution.

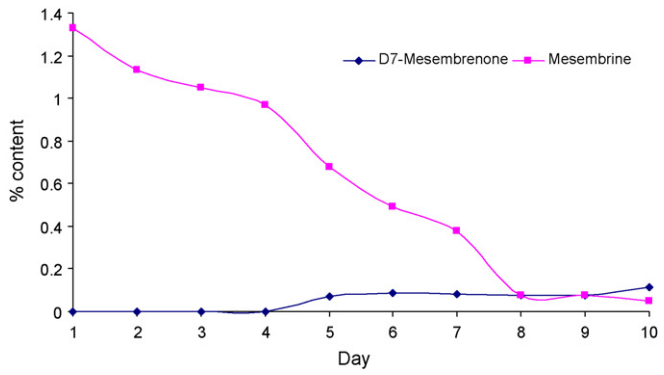

Fig. 5. Fermentation of *Sceletium tortuosum*. Study 1, showing loss of mesembrine and increase in Δ^7 mesembrenone (10 day study).

4. Conclusions

Whilst previous fermentation studies of *Sceletium* indicated changes in 4′-*O*-demethylmesembrenol and mesembrine content, the resulting transformation was reported to yield an unspecified mesembrenone which implied that the compound was Δ^4mesembrenone and which was incorrectly assigned as such in a publication by Smith et al., 1998. Furthermore, the published literature suggests that unspecified mesembrenone and the alkaloidal changes resulted from crushing and bruising the plant. The present study, however, has revealed that the fermentation process unequivocally transforms mesembrine to Δ^7mesembrenone and requires an aqueous environment together with the presence of light to facilitate such a transformation. This was achieved by

monitoring the alkaloidal changes over the entire period of the fermentation process and by quantifying and identifying the specific alkaloids. The results from the fermentation studies were also substantiated by conducting experiments using MHCl under different conditions of light and temperature.

The HPLC-PDA and LC–MS analytical methods developed and applied during this study are novel and were validated using qualified reference substances by isolating and purifying the specific alkaloids which are not commercially available.

These studies indicate that if mesembrine is the alkaloid that is purported to cause the claimed biological activity/pharmacological effect, then the claims of more effective material due to fermentation are questionable. Whereas our results confirm the transformation of mesembrine, the mechanism whereby this change occurs requires confirmation. Although the presence of water and sunlight appear necessary, the suggested enzymatic activity during fermentation of *Sceletium* as reported by Smith et al. (1998) needs further investigation. Such a study should involve the addition of a specific enzyme inhibitor during the fermentation process and subsequent monitoring of the content of mesembrine and Δ^7mesembrenone.

Acknowledgements

The authors acknowledge the National Research Foundation, South Africa for financial assistance and Mrs. M. Schwagmann, Dr. R. Keyzers, Dr. S. Ramdhani and Dr. S. Porches for their assistance during this study.

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