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Phytochemical Characterization and Biological Activities of Stenomesson miniatum Bulb Extract, a Medicinal Plant of the Andes.

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Phytochemical Characterization and Biological Activities of *Stenomesson miniatum* Bulb Extract, a Medicinal Plant of the Andes

Mariacaterina Lianza [a,*], Carmela Fimognari [a], Jean-Marc Nuzillard [b], Ferruccio Poli [c], Jean-Hugues Renault [b], Francesca Bonvicini [c], Giulia Greco [d] and Eleonora Turrini [a]

[a] Department for Life Quality Studies, University of Bologna, Rimini, Italy
[b] CNRS, ICMR UMR 7312, University of Reims Champagne-Ardenne, Reims, France
[c] Department of Pharmacy and Biotechnology, University of Bologna, Bologna, Italy
[d] Department of Chemistry, University of Bologna, Bologna, Italy
* Correspondence: mariacaterina.lianz3@unibo.it

Abstract: Fresh bulbs of *Stenomesson miniatum*, a plant belonging to the Amaryllidaceae family with a poorly investigated phytochemical profile, were traditionally employed by Andean healers to treat tumors and abscesses. The aims of this study were to characterize the extract from the bulbs of *S. miniatum* and to test its cytotoxic and antibacterial potential. A previous structural study of the major extract constituents was extended to include the minor components by means of ^{13}C-NMR-based dereplication. Cytotoxic activities were evaluated on A431 human epidermoid carcinoma cells through a metabolic assay and on Jurkat human acute T-leukemia cells through a cell-impermeant fluorescent nuclear probe. Antibacterial assays were carried out against *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus pyogenes* by using a standardized broth microdilution method. Eleven known Amaryllidaceae alkaloids were identified together with another compound determined as being an extraction artefact. The alkaloid-enriched extract showed good cytotoxic activity against both tumor cell lines, reaching an IC_{50} of 3.3 μg/mL against A431 cells and of 10.9 μg/mL against Jurkat cells. Biological assays carried out on single fractions showed that activity can be attributed to the presence of pretazettine and haemanthamine. Conversely, no antibacterial activities were recorded for any of the samples.

Keywords: *Stenomesson miniatum*; Amaryllidaceae alkaloids; *Urceolina peruviana*; ^{13}C-NMR-based dereplication; A431 human epidermoid carcinoma; Jurkat cells; artefact; Andean traditional medicine

1. Introduction

1.1. Stenomesson miniatum and the Amaryllidaceae alkaloids in Andean traditional medicine

The use of plants belonging to the Amaryllidaceae family is fairly widespread in the traditional medicines of the areas where these species are prominent, such as the Mediterranean basin, South Africa, and Andean South America (Nair and van Staden 2013). The principal bioactive compounds produced by these plants are isoquinoline alkaloids, peculiar to the species of the subfamily Amaryllidoideae (Bastida et al. 2006). The Amaryllidaceae alkaloids were proved to be responsible for most of the medicinal properties exhibited by these plants, since they are endowed with several types of biological activities, including cytotoxic (Lianza et al. 2020), anticholinesterase (Ee et al. 2004), antiviral (Chen et al. 2020), and antibacterial activities (Nair et al. 2017). The genus *Stenomesson*, native to the Andean regions of South America, has been used for medicinal purposes since pre-Columbian times by the Incas and later by other indigenous populations (Bastien 1982; Lévi-Strauss 1952; Nair 2019) . However, many species of this genus remain unstudied in terms of their chemical and biological features. *Stenomesson miniatum* (Herb.) Ravenna is one of these. It was initially classified as belonging to the genus *Urceolina*, due to the urceolate appearance of the corolla and the ventricose perianth (Meerow 1985), for this reason it is widely known as *Urceolina peruviana* (C.Presl) J.F.Macbr.

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which is a synonym for this species (https://wfoplantlist.org/plant-list/ accessed on March 2023). *S. miniatum* is a bulbous perennial plant, which grows spontaneously in the Andean regions of Bolivia and Peru above 2,000 m and up to 3,500 m a.s.l. (Meerow et al. 2015). The itinerant healers of the Andes, named Kallawaya, employed the bulbs of *S. miniatum* to treat tumors and abscesses, administering an ointment for topical use made from fresh grated bulbs and llama or pig fat (Girault 2018). To the best of our knowledge, only one paper was published regarding the phytochemical characterization of *S. miniatum* bulbs. In 1957, Boit and Döpke analyzed the alkaloid composition of three-year old bulbs grown in Holland; they reported the identification of tazettine, haemanthamine, and lycorine, and two other alkaloids corresponding to the chemical structures of albomaculine and nerinine (Boit and Döpke 1957). Hence, a more detailed characterization of this species is presented in this study.

1.2. Dereplication approach for phytochemical characterization

Obtaining pure compounds from a plant extract is difficult, time-consuming, and costly due to the wide range of required experimental techniques. Hence, in recent years, natural product chemists have accelerated drug discovery processes through the development of dereplication approaches (Gaudêncio and Pereira 2015). The term dereplication refers to a process of quick identification of known chemotypes (Hubert et al. 2017), avoiding, at least in part, purification processes, thus reducing times, costs and the generation of hazardous pollutants. Alkaloids from Amaryllidaceae plants have been intensively studied in the last few decades and, to date, more than 600 compounds have been isolated (Knolker 2020). Hence, Amaryllidoideae species are well suited for this type of approach. Our method relied on the idea that dereplication of natural products is best achieved by collecting taxonomic, structural, and spectroscopic data altogether in a database. Unfortunately, such free databases do not exist. Our previous publication explained the features and the construction of databases starting from the KNApSAcK website, the Universal Natural Product Database (UNPD), and COCONUT taking some of the alkaloids of *S. miniatum* as examples (Lianza et al. 2021). The complete characterization of the bulb extract is presented in this article. The use of ^{13}C NMR spectroscopy as tool for dereplication brought up an issue concerning the scarcity of freely available reference experimental spectroscopic data, a problem solved by means of prediction software (e.g. ACD/Labs software). Tools that rely on the matching of experimental data with predicted ones depend on the reproducibility of the former. The NMR spectra of part of the identified alkaloids were recorded in hexadeuterated dimethylsulfoxide (DMSO-d_6) and deuterated chloroform ($CDCl_3$) for chemical shift comparison. Considering the interesting ethnobotanical usage and the poor phytochemical knowledge of this species, the aims of this study were to characterize the alkaloid-enriched bulb extract of *S. miniatum* and to determine if its medicinal properties could be ascribed to the presence of Amaryllidaceae alkaloids. The topical use of the ointment prepared by indigenous healers from fresh bulbs suggested a possible action against skin tumors and bacteria causing skin infections. Hence, cytotoxic activities against A431 human epidermoid carcinoma and, subsequently, against an in vitro model of a hematological tumor, i.e. Jurkat leukemia cells, as well as antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus pyogenes* were assessed.

2. Materials and Methods

2.1. Plant material

Fresh bulbs of *S. miniatum* (Herb.) Ravenna (1090.3 g) were purchased in August 2019 at the plant nursery Quatro Estaciones in Cochabamba (Bolivia). For further verification some bulbs were grown, and the flowering plants were identified by Dr. Umberto Mossetti (Botanical Garden of the University of Bologna). A voucher specimen of these plants (BOLO0602041) is deposited in the Herbarium of the University of Bologna. The plant name was checked with http://www.worldfloraonline.org/ accessed in April 2022, which provides more up-to-date information than http://www.theplantlist.org/.

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2.2. Chemicals

Acetonitrile (CH_3CN), methyl-tert-butyl ether (MtBE), triethylamine (TEA), methanol (MeOH), sulfuric acid (H_2SO_4), ethyl acetate (EtOAc) and chloroform ($CHCl_3$) were purchased from Carlo Erba Reactifs SDS (Val de Reuil, France). DMSO-d_6 and $CDCl_3$ were purchased from Eurisotop (Saclay, France).

2.3. Dereplication approach

The dereplication approach applied in this study was composed by several steps including different techniques. The crude extract was cleared from non-alkaloid compounds by acid-base liquid-liquid partition. The pre-purified extract was fractionated by Centrifugal Partition Chromatography (CPC) to obtain chemically simplified fractions which were analyzed by Ultra Performance Liquid Chromatography coupled with High Resolution Mass Spectrometry (UPLC-HRMS) and by 1D and 2D NMR spectroscopy. The matching between the ^{13}C NMR data and the molecular formula of the compound under investigation with those stored in databases (*e.g.* KnapsackSearch, CSEARCH) allowed the rapid identification of already reported alkaloids. The chemical structures were validated by the analysis of the NMR spectra.

2.3.1. Preparation of the alkaloid-enriched extract

The alkaloid-enriched extract was prepared according to the protocol proposed by Renault et al. (2009) with slight modifications. The freeze-dried crushed bulbs (220 g) were moistened with NH_4OH 2.5 M and macerated in 4 L of EtOAc for three days. The extractive solution was collected by lixiviation and a further 4 L of EtOAc were added for maceration for other two days, then the solution was concentrated to 1 L. Solid-liquid extraction by EtOAc instead of CH_2Cl_2 was chosen to avoid the known reaction of the latter with tertiary amine with halogenated solvents (Maltese et al. 2009). The EtOAc solution was extracted with 0.2 L (x3) and 0.1 L (x3) of H_2SO_4 0.6 M, the aqueous phase was basified with NH_4OH 7.5 M until pH 10 and extracted with 0.2 L (x3) and 0.1 L (x3) of $CHCl_3$. Finally, the organic phase was washed with water until pH 7 and the solvent evaporated under reduced pressure to leave 2.7 g of alkaloid extract.

2.3.2. Centrifugal Partition Chromatography

Centrifugal partition chromatography (CPC) was carried out on 1 g of the bulb extract as reported by Lianza et al. (2021) obtaining thirteen fractions (A1-A13), whose masses and extraction yields are reported in Table S1 of Supplementary Information 2 (SI2). Fraction 12 was further fractionated using a device adapted to its low mass (99.0 mg), i.e. a FCPC-A200 column with 202 mL inner volume (Kromaton Technology, Angers, France). The column was composed of 21 circular partition disks, each engraved with 40 twin-cells of 0.24 mL. The liquid phases were pumped by a preparative 1800 V7115 pump (Knauer, Berlin, Germany) and the sub-fractions collected by a Labocol Vario 4000 (Labomatic Instruments, All-schwil, Switzerland). The biphasic solvent system was the same as the one used for extract fractionation, i.e. MtBE: CH_3CN: H_2O (5:2:3, v/v/v). The concentration of the retainer (1.5 mM H_2SO_4), and of the displacer (2 mM TEA), were adapted to the small sample mass.

2.3.3. UPLC-HRMS

The Ultra Performance Liquid Chromatography coupled with High Resolution Mass Spectrometry (UPLC-HRMS) analyses were performed as already reported by Lianza et al. (2021).

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2.3.4. NMR

NMR analyses for spectra recording in DMSO-d_6 were performed according to Lianza et al. (2021). For the analysis in $CDCl_3$, the central resonance (triplet) was set at δC 77.16 for ^{13}C NMR spectrum referencing and at δH 7.26 for ^{1}H NMR spectrum referencing.

2.4. Cytotoxic activity

2.4.1. Cell cultures

Authenticated A431 human epidermoid carcinoma cells and Jurkat cells (both provided by LGC Standards, Teddigton, Middlesex, UK) were propagated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin solution (all purchased by Euroclone, Pero, Italy). Cells were grown at 37°C in a humidified incubator with 5% CO_2.

2.4.2. Cell viability assays

The alkaloid-enriched extract and its fractions (A2, A4, A6, A7, A8, A9, A10, A11, A12, and A13) were dissolved in DMSO at a final concentration of 50 mg/mL. Cells were treated with increasing concentrations of extract or its fractions (0.005 – 0.500 mg/mL) for 24, 48 and 72 h. The analysis of cell viability was performed using two different tests: the cell-impermeant fluorescent nuclear probe SytoxTM green (Thermo Fisher Scientific, Waltham, MA, USA) for the suspended Jurkat cells and a metabolic assay, using 4-methylumbelliferyl heptanoate (MUH, Merck, Darmstadt, Germany), for the adherent A431 cells. Fluorescence was measured with a Guava EasyCyte 6-2L flow cytometer (Merck) or Victor X3 microplate reader (Perkin Elmer, Walthman, MA, USA), respectively. The half maximal inhibitory concentration (IC_{50}) was calculated and normalized to the effect of the added DMSO. IC_{50} was calculated by interpolation from a dose-response curve [non-linear regression, log(inhibitor) vs normalized response].

2.4.3. Statistical analysis

Results are shown as means ± SEM of at least two different experiments. Significant differences among treatments were assessed by two-way analysis of variance (ANO-VA), using Dunnett as post-hoc test. GraphPad Prism 6 (Inc. La Jolla, CA, USA) was used for the statistical analysis and $p < 0.05$ was considered significant.

2.5. Antibacterial activity

2.5.1. Preparation of extract and fractions for antibacterial activity

For microbiological assays, the extracts were solubilized in DMSO at 20 mg/mL to obtain stock solutions, which were stored at 4°C until use.

2.5.2. Bacterial strains and antibacterial assay

Staphylococcus aureus ATCC 25293, *Staphylococcus epidermidis* (ATCC 12228), and *Streptococcus pyogenes* (ATCC 19615) were selected as representative strains to test the antibacterial properties of plant extracts by using a standardized microdilution broth method in a 96-well plate (Mandrone et al. 2019) according to the guidelines established by several international committees (Clinical and Laboratory Standards Institute, European Committee on Antimicrobial susceptibility testing). Bacterial strains were routinely cultured in 5% blood agar at 37°C. For experiments, the bacterial suspensions were prepared in PBS (phosphate buffer saline), adjusted at 0.5 McFarland, corresponding to 10^8 colony forming units (CFU)/mL, and subsequently diluted 1:200 in Brain Heart Infusion Broth (Sigma-Aldrich); they were incubated with the extract and its fractions at 200 µg/mL. A few wells were reserved in each microplate for negative (no inoculum added) and positive growth controls. The microplate was incubated at 37 °C and bacterial growth was monitored by measuring

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the optical density at 630 nm (Multiskan Ascent microplate reader, Thermo Fisher Scientific Inc., Waltham, USA). Percentage values of bacterial growth were determined relative to the positive control.

3. Results and Discussion

3.1. Phytochemical characterization by dereplication of S. miniatum bulb extract

Phytochemical characterization was conducted on chemically simplified fractions of the alkaloid-enriched extract, obtained from Centrifugal Partition Chromatography (CPC), by matching their ^{13}C NMR spectra and molecular formula with those found in databases (*e.g.* KnapsackSearch, CSEARCH), and validating the chemical structures by the analysis of ^{1}H NMR and 2D NMR spectra. The 2D NOESY spectra and the measurement of ^{1}H-^{1}H coupling constants provided data for the assessment of relative configurations of asymmetric centres and the tentative discrimination of inequivalent hydrogen atoms in methylene groups. Thirteen alkaloids (including two epimers) were identified (Figure 1), twelve of them being already known (compounds 1-10, 11A, 11B).

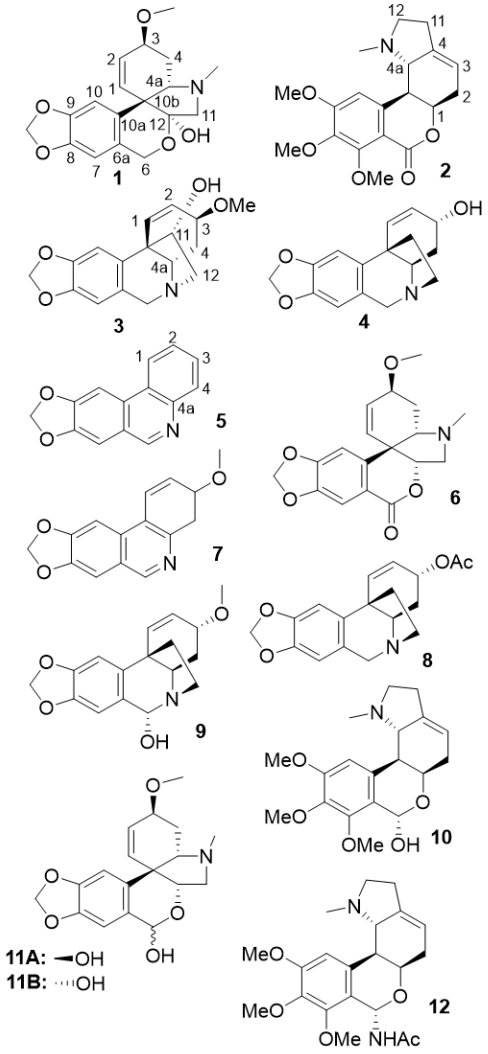

Figure 1. Chemical structures of the alkaloids identified in the *Stenomesson miniatum* bulb extract: tazettine **1**, albomaculine **2**, haemanthamine **3**, crinine **4**, trisphaeridine **5**, 3-epimacronine **6,** 3-methoxy-8,9-methylenedioxy-3,4-dihydrophenanthridine **7**, crinine acetate **8**, 6α-hydroxybuphanisine **9**, nerinine **10**, β-pretazettine **11A**, α-pretazettine **11B**, 6-dehydroxy-6-acetamido-nerinine **12**.

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CPC in pH-zone refining mode is a chromatographic separation technique which allows the purification of compounds whose electric charge depends on pH, as in the case of alkaloids. The use of an acid retainer (*e.g.* H_2SO_4) in the aqueous stationary phase and a basic displacer (*e.g.* TEA) in the organic mobile phase enables the separation of the alkaloids by contiguous blocks arranged according their pK_a and partition coefficients (Renault et al. 1999). Collecting fractions in the centre of the blocks leads to the recovery of high-purity fractions (Kotland et al. 2016). A CPC run yielded i) purified alkaloids in fraction A4, A7 and A9, ii) highly chemically simplified fractions (*i.e.* mainly enriched in one alkaloid) A3, A5, and iii) mixtures in fractions A2, A6, A8, A10, A11, A12 and A13. Our previous publication (Lianza et al., 2021) reported the extraction, fractionation, and the detailed structure elucidation of compounds **1—3** namely tazettine, albomaculine and haemanthamine, isolated at a high purity level. Lianza et al. (2021) also reported the structure elucidation of crinine (compound 4) and trisphaeridine (compound **5**) identified in mixture fractions. The structures of compounds **6—12**, identified in mixture fractions, were not reported in our previous work. UPLC-HRMS analysis provided the molecular formula of each alkaloid (data reported in SI3), allowing the field of investigation to be narrowed down for ^{13}C NMR-based dereplication. Table 1 indicates the fractions from which alkaloids were identified, and the reference used for ^{13}C NMR-based dereplication. Among the extract fractions, a complex and abundant one (A12) showed a compound with an NMR profile that was not compatible with any of those previously reported, as attested by its absence from the SciFinder^n database (https://scifinder-n.cas.org). Hence, it was subjected again to CPC in order to obtain simpler fractions, among which A12_8 was the most useful for compound identification. The ^1H NMR spectrum of compound **12** showed an isolated doublet at 8.84 ppm for a hydrogen atom that was not bound to a carbon atom, according to the HSQC spectrum. The exploration of the neighborhood of this hydrogen atom by means of the HMBC spectrum indicated the presence of the acetamido group. The COSY correlation of the NH signal provides the entry point into the nerinine structure element via its position 6. The compound was identified as 6-dehydroxy-6-acetamido-nerinine, which was hitherto never reported in the literature. Amaryllidaceae alkaloids with two nitrogen atoms are rare; some examples are the plicamine and secoplicamine type, together with some of those belonging to the group of miscellaneous and unclassifiable compounds (Berkov et al. 2020; de Andrade et al. 2012). One of the most widely accepted hypotheses for the formation of dinitrogenous alkaloids is the reaction between an intermediate of the biogenic pathway of an alkaloid with one nitrogen atom, and an amino acid, which provides the second nitrogen atom. For example, a key aminoaldehyde intermediate for the biogenesis of crinine and tazettine type alkaloids probably reacts with tyramine for the formation of plicamine, secoplicamine, and obliquine (Ünver et al., 1999). In the structure of compound **12**, incorporation of an amino acid could not be detected; it is more likely, therefore, that a reaction of the OH group at position 6 of nerinine with the reagents of the extraction process took place. The simultaneous presence of ammonium hydroxide (NH_4OH) and ethylacetate (EtOAc) for five days during extraction can explain the formation of compound **12** from nerinine according to Figure 2. Despite being a useful aqueous base for extraction of Amaryllidaceae alkaloids, NH_4OH reacts with carbonyl groups to give condensation products (Maltese et al., 2009); the typical hemiacetals in the structure of alkaloids or solvents with a carbonyl group, such as EtOAc, are therefore particularly susceptible. Thus, compound **12** was classified as an extraction artefact.

Table 1. Fraction composition, identified alkaloids in different fractions (first identification) of the *Stenomesson miniatum* bulb extract and bibliographic reference for ^{13}C NMR-based dereplication.

FRACTION	Fraction composition	Identified Alkaloid	Reference for ^{13}C NMR-based dereplication
A1	-	-	-
A2	tazettine; trisphaeridine;	Trisphaeridine **5**; 3-epimacronine **6**;	(Viladomat et al. 1997)

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	3-epimacronine; 3-methoxy-8,9-methylenedioxy-3,4-dihydrophenanthridine	3-methoxy-8,9-methylenedioxy-3,4-dihydrophenanthridine **7**	(Viladomat et al. 1990) (Hohmann et al. 2002)
A3	tazettine; trisphaeridine		
A4	tazettine	tazettine **1**	(Knolker 2020)
A5	tazettine; crinine acetate		
A6	crinine acetate; albomaculine	crinine acetate **8**	(Ali et al. 1986)
A7	albomaculine	albomaculine **2**	(de Andrade et al. 2014)
A8	albomaculine; 6α-hydroxybuphanisine; haemanthamine	6α-hydroxybuphanisine **9**	(Frahm et al. 1985)
A9	haemanthamine	haemanthamine **3**	(Viet Nguyen et al. 2019)
A10	haemanthamine; nerinine	nerinine **10**	(de Andrade et al. 2014)
A11	crinine; α-pretazettine	crinine **4**	(Viladomat et al. 1995)
A12	α-pretazettine; β-pretazettine; 6-dehydroxy-6-acetamido-nerinine	β-pretazettine **11A** α-pretazettine **11B** 6-dehydroxy-6-acetamido-nerinine **12**	(Baldwin and Debenham 2000) (Kobayashi et al. 1980) -
A13	β-pretazettine; α-pretazettine; 6-dehydroxy-6-acetamido-nerinine		

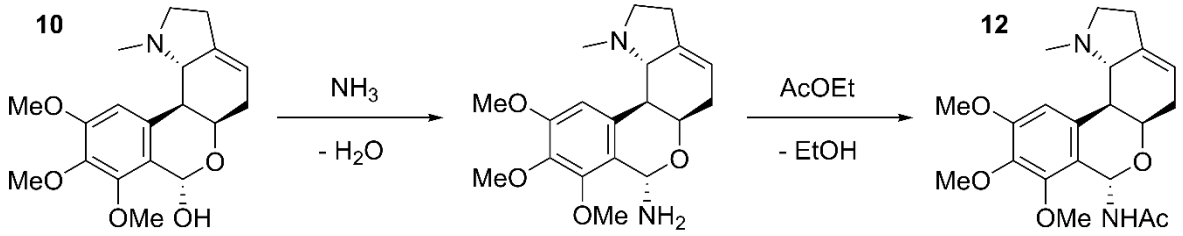

Figure 2. A likely mechanism for the formation of compound **12** from nerinine (compound **10**) during the alkaloid extraction process.

The description of the NMR spectra of all compounds is available from the zenodo.org archive in three forms. The first form is the traditional data table as published in chemistry journals, the second one is a text file that is structured according to the guidelines of the NMReDATA organization (Kuhn et al., 2021) for good human- and computer- readability, and the third one is a structure drawing, reproduced on a larger scale for a good readability by humans and with chemical shift values reported for each hydrogen and carbon atom. Accession details are reported in the Supplementary Information file 1 (SI1).

The spectra of all fractions obtained by CPC were analyzed by NMR using DMSO-*d6* as solvent. This choice was motivated by the systematic use of this solvent in the authors' laboratory as it has a strong dissolution ability for a very wide range of analytes. Moreover, DMSO-*d6* facilitates the detection of the ^{1}H NMR signal of exchangeable nuclei, thus allowing an efficient structural analysis.

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However, fractions A4, A7, A9, and A11 containing compounds **1—4** were also analyzed in $CDCl_3$, a solvent that is widely used for the analysis of Amaryllidaceae alkaloids. The ^{13}C NMR chemical shifts recorded using the two solvents is presented in Supplementary Information file 1 (SI1). Our experimental values were in good agreement with those available in previously published reports (for all compounds except **7**, **11B,** and **12**). The good reproducibility of NMR chemical shift values, regardless of the solvent used, confirms the high reliability of compound search in databases from ^{13}C NMR data.

3.2. Biological activities of S. miniatum bulb extract

The extract and some of the fractions were subjected to the fore-mentioned biological assays. Among the 13 fractions obtained from CPC fractionation, A1 had a scarce mass (4 mg); thus, the NMR analysis was difficult to interpret due to the low concentration of alkaloids, while fractions A3 and A5 contained tazettine in a less pure form than the one in fraction A4. Consequently, these fractions were not tested for biological activities.

3.2.1. Cytotoxic activities against A431 human epidermoid carcinoma cells

The extract and part of the fractions were tested against human epidermoid A431 carcinoma cells by measuring cell viability after 24, 48 and 72 h of treatment. The extract was slightly more potent than the other samples, with an IC_{50} of 3.3 μg/mL after 72 h. At the same time point, the IC_{50} calculated for fractions A8, A9, A10, A11, A12, and A13 was in the range 3.7-8.2 μg/mL (Table 2).

Table 2. IC_{50} values in μg/mL calculated after treatment of A431 cells with increasing concentrations of the extract or its fractions for 24, 48 or 72 h.

Sample	**IC_{50} 24h**	**IC_{50} 48h**	**IC_{50} 72h**
extract	9.1	6.7	3.3
A2	347.1	297.5	232.1
A4 (tazettine)	901.3	1171.0	869.2
A6	394.0	419.0	412.9
A7 (albomaculine)	201.5	251.5	168.7
A8	10.1	7.1	5.1
A9 (haemanthamine)	7.6	5.4	3.7
A10	16.1	13.2	5.2
A11	9.9	10.3	8.2
A12	5.7	4.3	5.3
A13	6.4	4.9	3.8

The biological activity of a plant extract and of its fractions depends on synergism and antagonism. Action additivity among the components of a mixture may depend on its composition and on the relative concentrations of the individual components (Catanzaro et al. 2018; Fimognari et al. 2012; Lenzi et al. 2018). This means that, even when both the extraction and fractionation protocols are provided, if fractions with different concentrations of individual components are obtained, different studies often yield different results in their evaluation of biological activity. However, this approach allows the identification of the most promising pool of molecules, discriminating them from fractions that show no activity. The alkaloid-enriched extract was predominantly composed of tazettine, haemanthamine, albomaculine, and crinine, with tazettine being the most abundant (Table S1 of Supplementary Information file 2). Tazettine is not a naturally occurring alkaloid, but an artefact formed under basic conditions by molecular rearrangement of chemically labile pretazettine during extraction (de Andrade et al. 2012; Kobayashi et al. 1980). Consequently, the major alkaloid present

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in the fresh bulbs of *S. miniatum*, used for medicinal purposes in ethnobotany, turned out to be pretazettine. Pretazettine was successfully tested against A431 tumor cells, resulting in an IC_{50} of 5.4 μM (Zupkó et al., 2009). Moreover, it exhibited cytotoxicity against Rauscher leukemia, AKR lymphoblastic leukemia, HeLa cervical adenocarcinoma, Lewis lung carcinoma and Ehrlich ascites carcinoma cells (Bastida et al., 2006). Among the fractions showing inhibition of A431 cell viability, A11, A12, and A13 contained pretazettine at various concentrations, mixed with other alkaloids, including crinine (A11), and 6-dehydroxy-6-acetamido-nerinine (A12 and A13). Three pure alkaloids, namely tazettine (A4), albumaculine (A7) and haemanthamine (A9), were also investigated. In our experimental model, tazettine did not show any significant activity, as previously reported (Masi et al., 2022). Albomaculine, tested for the first time against the A431 tumor cell line, was not found to be active either. Conversely, haemanthamine inhibited A431 cell viability with an IC_{50} of 3.7 μg/mL (12.3 μM) after a 72-h treatment. This result does not match with the one published by Masi et al. (2022), who found the compound to be inactive at 0.5-10 μM concentrations using the MTT cell viability assay. The different cytotoxic effect is not surprising and can be due to the higher concentrations of haemanthamine we tested in the present study compared to those used by Masi et al. Fractions A8 and A10, containing haemanthamine in mixture, were also effective. Considering that none of the other alkaloids identified in the mixture, namely albomaculine and 6-hydroxybuphanisine in A8, and nerinine in A10, exhibited significant cytotoxic activity in several tumor cell screenings (Nair et al. 2016; Nair and Van Staden, 2021), haemanthamine is probably responsible for part or all the effect of these fractions on A431 cell viability. Among the Amaryllidaceae alkaloids, haemanthamine has been singled out for its promising anti-cancer properties; in fact, it is a candidate lead for the development of drugs against several types of carcinomas and leukemia (Cahlíková et al., 2021). Overall, the bulb extract of *S. miniatum* showed a good cytotoxicity against A431 human epidermoid carcinoma cells. Conversion of the original pretazettine to the inactive tazettine during the extraction procedure certainly modified the cytotoxic potential of *S. miniatum* bulbs, which would be even higher under natural conditions.

3.2.2. Cytotoxic activities against Jurkat human acute T-leukemia cells

The alkaloid extract of *S. miniatum* and its fractions were tested against another type of cancer, a blood tumor (Table 3).

Table 3. IC_{50} values in μg/mL calculated after treatment of Jurkat leukemia cells with increasing concentrations of the extract or its fractions for 24, 48 or 72 h.

Sample	**IC_{50} 24h**	**IC_{50} 48h**	**IC_{50} 72h**
extract	124.6	31.4	10.9
A2	309.9	209.5	123.8
A4 (tazettine)	1373.0	857.8	881.9
A6	894.8	360.7	256.1
A7 (albomaculine)	1669.0	1073.0	446.1
A8	233.3	31.7	13.7
A9 (haemanthamine)	70.4	31.2	4.5
A10	292.3	53.7	13.9
A11	102.4	53.7	8.6
A12	119.3	16.4	5.1
A13	65.6	12.4	5.5

On the whole, the cytotoxicity exhibited by the alkaloid-enriched extract of *S. miniatum* and its fractions on blood tumor cells was lower than against A431 cells (Table 3). The extract inhibited the viability of Jurkat cells with an IC_{50} of 10.9 μg/mL after a 72-h treatment. The fraction consisting of pure haemanthamine (A9) was the most toxic, but the IC_{50} value was higher than that recorded for

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A431 cells (14.9 μM *versus* 12.3 μM). This alkaloid was already tested against Jurkat cells by Mc Nulty et al. who reported an IC_{50} of 14.9 μM, matching the one found in our experiments (McNulty et al., 2007). The same authors pointed out that the α 5-10b-ethano bridge and the hydroxyl group at C-11 of the chemical structure of haemanthamine are key structural features for the induction of selective apoptosis. The other haemanthamine-containing fractions were less effective than the total extract. Conversely, the fractions containing pretazettine (A11, A12, and A13) inhibited cell viability more than the extract. All experimental results are shown in Supplementary Information file 2 (SI2).

S. miniatum bulbs proved to be a source of pretazettine and haemanthamine, two Amaryllidaceae alkaloids endowed with potent cytotoxic activities, thus supporting their anticancer ethnobotanical use.

3.2.3. Antibacterial activities

The bulb extract of *S. miniatum* and the fractions were tested against bacteria triggering skin abscesses, namely *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes*. No sample showed appreciable activity at the tested concentration (200 μg/mL). Some of the alkaloids contained in the extract, namely tazettine, haemanthamine and crinine, were already tested against *S. aureus* and *S. epidermidis* without revealing antibacterial properties (Ločárek et al. 2015; Nair et al. 2017). The antibacterial action reported by the Kallawaya healers could be due to non-alkaloid metabolites contained in whole fresh bulbs. Amaryllidaceae plants produce other types of compounds, such as ceramides, which showed a moderate antibacterial activity against *S. aureus* (Wu et al., 2009), besides polyphenols, whose antibacterial action is widely recognized (Coppo and Marchese, 2014). Further studies could be conducted on the analysis of non-alkaloid compounds produced by this species.

4. Conclusion

The dereplication approach for the characterization of *S. miniatum* bulb extract allowed the rapid identification of the twelve known Amaryllidaceae alkaloids and a new one artefact. The phytochemical profiling led to the detection of various cytotoxic alkaloids to which the cytotoxic activity against the tested tumor lines can be ascribed. Hence, the ethnobotanical use of *S. miniatum* bulb extract as skin cancer treatment was corroborated by this study. Additional research could be conducted on antibacterial activity, focusing on non-alkaloid compounds produced by this species.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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Abbreviations

ANOVA	Analysis of variance
COCONUT	COlleCtion of Open Natural ProdUcTs
CPC	Centrifugal partition chromatography
KNApSAcK	Kurokawa Nakamura Asah personal Shinbo Altaf-Ul-Amin computer Kanaya
MtBE	Methyl tert-butyl ether
NMReDATA	NMR extracted data
RPMI	Roswell Park memorial institute
SEM	Standard error of the mean
TEA	Triethylamine
UNPD	Universal natural products database

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UPLC Ultra performance liquid chromatography

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