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

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Article

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Abstract: The fresh bulbs of *Stenomesson miniatum*, a plant belonging to 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 alkaloid extract from the bulbs of *S. miniatum* and test its cytotoxic and antibacterial potential. The alkaloid extract was characterized by dereplication using various techniques (CPC, NMR, UPLC-HRMS) and referring to in-home or online databases for spectroscopic data matching. 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 microdilution broth method 96-well plate. Eleven known Amaryllidaceae alkaloids were identified together with another compound determined as being an extraction artefact. The alkaloid extract showed good cytotoxic activity against both the tumor cell lines, reaching an IC_{50} of 3.3 µg/mL against A431 cells and of 10.9 µg/mL against Jurkat cells. The analysis of the fractions allowed the activity to be attributed to the presence of pretazettine and haemathamine. 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, skin cancer, Jurkat cells, artefact, Andean traditional medicine

1. Introduction

The use of plants belonging the Amaryllidaceae family is fairly widespread in the traditional medicines of the areas where these species are prominent, such as Mediterranean basin, South Africa and Andean South America [1]. The medicinal properties exhibited by these plants are mainly due to the presence of isoquinoline alkaloids, peculiar to species of the subfamily Amaryllidoideae [2]. These secondary metabolites are endowed with several types of biological activities, including cytotoxic [3], anticholinesterase [4], antiviral [5], and antibacterial activity [6]. The genus *Stenomesson,* disseminated in the Andean regions of South America, was used for medicinal practices since the times of Inca, and later by subsequent indigenous populations [7–9], however, many species remain unstudied both from chemical and biological point of view. Among these is *Stenomesson miniatum* (Herb.) Ravenna. It is widely known as *Urceolina peruviana* (C. Presl) J.F. Macbr., which is a synonym for this species (https://wfoplantlist.org/plant-list/ accessed on March 2023), actually it was initially classified as belonging to the genus *Urceolina*, an error due to the urceolate appearance of the corolla

 doi:10.20944/preprints202305.0921.v1

and the ventricose perianth morphology [10]. *S. miniatum* is a bulbous perennial plant which grows spontaneously in the Andean regions of Bolivia and Peru above 2000 meters up to an altitude of 3500 meters [11]. In 1957 Boit and Döpke analyzed the alkaloid composition of three years old bulbs grown in Holland, reporting the identification of tazettine, haemanthamine and lycorine, and two other alkaloids which correspond to the chemical structures of albomaculine and nerinine [12]. To the best of our knowledge, no further studies were published concerning its phytochemical characterization. The itinerant practitioners of the Andes, named Kallawaya, employed the bulbs of *S. miniatum* to treat tumors and abscesses. The healers, administered an ointment for topical use made from fresh grated bulbs and llama or pig fat [13]. Considering the interesting ethnobotanical usage and the poorly investigation on this species, the aims of this study were to characterize the alkaloid-enriched bulb extract of *S. miniatum* and to give a molecular basis of its uses. 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 turned their efforts to accelerate drug discovery processes trough the development of dereplication approaches [14]. The term dereplication refers to a process of quickly identifying known chemotypes [15], avoiding, at least in part, purification processes, thus reducing times, costs and the generation of hazardous pollutants. Amaryllidaceae alkaloids were intensively studied in the last few decades, to date more than six hundred compounds were isolated [16]. Hence, Amaryllidoideae species are well suited to this kind of approach as lot of compounds are already reported in scientific literature. The workflow and the tools of a dereplication method taking the major alkaloids of *S. miniatum* bulb extract as examples was described in our previous publication [17]. Our approach relied on the idea that dereplication of natural products is best achieved by collecting taxonomic, structural, and spectroscopic data altogether in a database. Hence, we explained the features and the construction of databases starting from the KNApSAcK website, the Universal Natural Product Database (UNPD), and the COCONUT database, including carbon-13 NMR spectroscopy data. The structure of minor compounds of *S. miniatum* 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/Lab software). Tools that rely on the matching of experimental data with predicted ones depend on the reproducibility of the former. Hence, we investigated how spectra were influenced by the nature of the solvent and how this impacted dereplication. At this end, the NMR spectra of part of the identified alkaloids were recorded in hexadeuterated dimethylsulfoxide (DMSO-*d6*) and deuterated chloroform ($CDCl_3$) for chemical shift comparison. The topical use of the ointment prepared by the indigenous healers from the fresh bulbs, suggested a possible action against skin tumors and bacteria triggering skin infections. Hence, the cytotoxic activities against A431 human epidermoid carcinoma were determined and the antibacterial activity tests against *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes* were carried out. The bulb extract and part of the fractions showed cytotoxic activities against A431 tumor cells; thus, the samples were also tested against an *in vitro* model of hematological tumor, *i.e.* Jurkat leukemia cells, showing again a cytotoxic effect. Conversely, no antibacterial activities were found for any of the samples.

2. Results and Discussion

2.1. Phytochemical characterization of S. miniatum bulb extract

Phytochemical characterization was conducted on chemically simplified fractions of the alkaloid-enriched extract, obtained by Centrifugal Partition Chromatography (CPC). The fractions 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 allowed the identification of already reported alkaloids, whose structures were validated by the analysis of ^{1}H NMR and 2D NMR spectra. Twelve alkaloids were identified (Figure 1) eleven of them being already known (compounds **1-11**).

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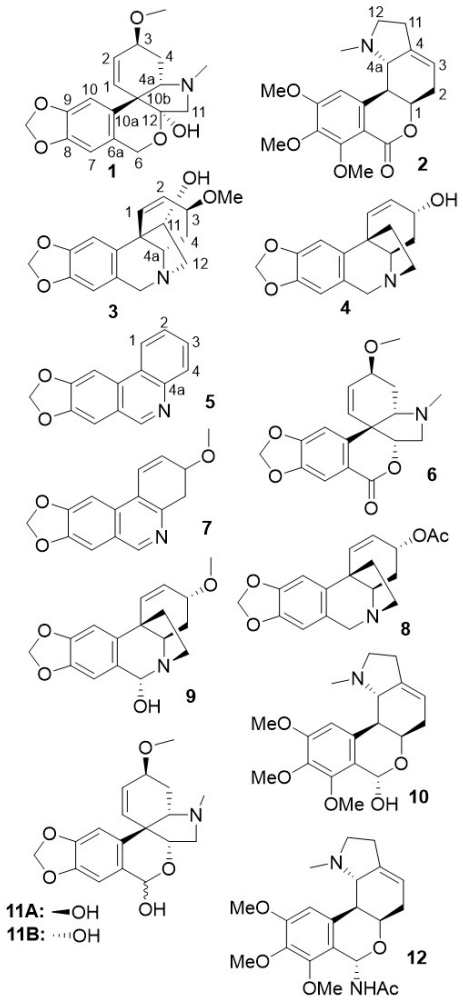

Figure 1: Chemical structures of the alkaloids identified in *S. miniatum* bulb extract: tazettine **1**, albomaculine **2**, haemanthamine **3**, crinine **4**, trisphaeridine **5**, 3-epimacronine **6,** 3-methoxy-8,9-methylenedioxy-3,4-dihydrophenanthridine **7**, crinine acetate **8**, 6α-hydroxybuphanisine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

CPC in pH-zone refining mode is a chromatographic separation technique which allows the purification of compounds whose electric charge depend on pH value, 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 [18]. Collecting fractions in the centre of the blocks leads to the recovery of high purity fractions [19]. A CPC run yielded the pure fractions A3, A4, A5, A7, A9 and mixtures in fractions A2, A6, A8, A10, A11and A12. Our previous publication [17] reported the extraction, fractionation, and the detailed structure elucidation of compounds **1—3** namely tazettine, albomaculine and haemanthamine, isolated at a high purity level. Reference [17] also reported the structure elucidation of crinine (compound **4**) and trisphaeridine (compound **5**) identified in mixture fractions. The structures of compounds **6—12** were not reported in our previous work and were established by means of a conventional approach that relied on the analysis of two-dimensional (2D) COSY, HSQC and HMBC spectra for the tracing of the compound planar formula. The 2D NOESY spectra and the measurement of ^1H-^1H coupling constants provided data for the assessment of relative configurations of asymmetric centres and the tentative discrimination of inequivalent

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hydrogen atoms in methylene groups. Table 1 indicates from which fractions the alkaloids were identified, and the used reference for ^{13}C NMR-based dereplication.

Table 1. Reference of fraction extract and bibliographic reference for the alkaloids identified in *S .miniatum* bulb extract

Compound	Fraction	Reference
tazettine	A4	[16]
albomaculine	A7	[20]
haemanthamine	A9	[21]
crinine	A11	[22]
trisphaeridine	A2	[23]
3-epimacronine	A2	[24]
3-methoxy-8,9-methylenedioxy-3,4-dihydrophenanthridine	A2	[25]
crinine acetate	A6	[26]
6α-hydroxybuphanisine	A8	[27]
nerinine	A10	[20]
β-pretazettine	A12_8	[28]
α-pretazettine	A12_8	[29]
6-dehydroxy-6-acetamido-nerinine	A12_8	-

Among the mixture fractions, a complex and abundant one (A12) showed a compound with an NMR profile not compatible with any of those already reported, as attested by its absence from the SciFinder^n database (https://scifinder-n.cas.org). Hence fraction A12 was submitted again to CPC resulting in 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 neighbourhood 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 never reported in literature. Amaryllidaceae alkaloids with two nitrogen atoms are rare; some examples are those of plicamine and secoplicamine type, together with some of the group of the miscellaneous and unclassifiable compounds [14,15]. 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 [32]. In the structure of compound **12**, it is not possible to recognize the incorporation of an amino acid; it is more likely that a reaction of the OH group at position 6 of nerinine with the reagents of the extraction processes took place. The simultaneous presence of ammonium hydroxyde (NH_4OH) and ethylacetate (EtOAc) for 5 days during extraction can explain the formation of compound **12** from nerinine according to Figure 2.

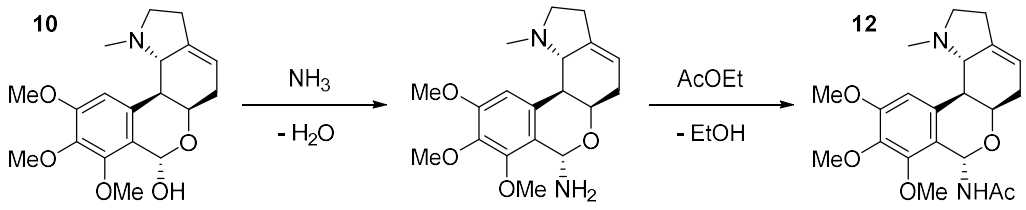

Figure 2. A likely mechanism for the formation of compound **12** from nerinine (compound **10**) during alkaloid extraction.

 doi:10.20944/preprints202305.0921.v1

Despite being a successful aqueous base for Amaryllidaceae alkaloids extraction, NH_4OH reacts with carbonyl group to give condensation products [33], the typical hemiacetals in the structure of alkaloids or solvents with carbonyl group, as EtOAc, are therefore particularly susceptible. Thus compound **12** was classified as an extraction artefact. 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 [34] for good human- and computer- readability, and the third one is a structure drawing reproduced at large 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 analysed 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 ^1H NMR signal of exchangeable nuclei, thus offering possibilities for an efficient structural analysis. However, fractions A4, A7, A9, and A11 containing compounds **1—4** were also analysed in $CDCl_3$, a solvent that is widely used for the analysis of Amaryllidaceae alkaloids throughout the literature. A comparison between the ^{13}C NMR chemical shifts of these samples recorded in the two solvents is presented in the Supplementary Information file 1 (SI1). The comparison between our experimental values and those from literature for all compounds for which this is possible (all but **7**, **11B** and **12**) is also provided and shows a very good agreement. The good reproducibility of experimental NMR chemical shift values, independently of solvent, confirms the high reliability of compound search in databases from ^{13}C NMR data. Access to spectroscopic data of all the identified alkaloids is described in the Supplementary Information file 1 (SI1).

2.2. Biological activities of S.miniatum bulb extract

The extract and part of the fractions were subject to the biological assays to assess the properties mentioned in the ethnobotanical uses. Among the thirteen fractions obtained from CPC fractionation, A1 had a scarce mass (4 mg) and the NMR analysis resulted of difficult interpretation due to the low concentration of the alkaloids, while fraction A3 and A5 consisted of tazettine less pure than that collected in fraction A4, thus these fractions were not tested for biological activities.

2.2.1. Cytotoxic activities against A431 human epidermoid carcinoma cells

The extract and part of the fractions were tested against human epidermoid A431 carcinoma cells, 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 of treatment. At the same time point, the IC_{50} calculated for the 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

 doi:10.20944/preprints202305.0921.v1

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 [29–31] . Thus, relating the cytotoxic activity to a particular alkaloid rather than to another one is not straightforward. The alkaloid enriched extract was predominantly composed by tazettine, haemanthamine, albomaculine and crinine, with tazettine being the most abundant as reflected by the extraction yields reported for each fraction in Table S2 of Supplementary Information file 2 (SI2). Tazettine is not a naturally occurring alkaloid, but an artefact formed under basic conditions by molecular rearrangement of chemically labile pretazettine during extraction [5,14]. Consequently, the major alkaloid produced in the fresh bulbs, which are mentioned in ethnobotanical use, turned out to be pretazettine. Pretazettine was successfully tested against A431 tumor cells, resulting in an IC_{50} of 5.4 μM [38]. Moreover, it exhibited cytotoxicity against Rauscher leukemia, AKR lymphoblastic leukemia, HeLa cervical adenocarcinoma, Lewis lung carcinoma and Ehrlich ascites carcinoma cells [2]. Among the fractions showing inhibition of A431 cells viability, A11, A12 and A13 contained pretazettine at diverse concentrations and 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 investigated. In our experimental model tazettine did not show any significant activity, as already reported in literature [39]. Albomaculine was not found to be active either, this alkaloid was tested for the first time against A431 tumor cell line. Conversely, haemanthamine inhibited A431 cells viability with an IC_{50} of 3.7 μg/mL (12.3 μM) after a 72h treatment. This result does not match with those published by Masi et al. [39], who deemed the compound inactive testing it thought MTT assay at 0.5-10 μM. 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, resulted endowed with notable cytotoxic activity in several tumor cell screening [41,42], probably haemanthamine is responsible for part or all the effect of these fractions on A431 cell viability. Among the Amaryllidaceae alkaloid haemanthamine has been singled out for its promising anti-cancer properties, in fact it is a candidate lead for the development of anti-cancer drugs being active against several types of carcinomas and leukemia [42]. Overall, the bulb extract of *S. miniatum* showed a good cytotoxicity against A431 human epidermoid carcinoma cells. The conversion of the original pretazettine to tazettine certainly modified the cytotoxic potential of *S. miniatum* bulbs, which would be even higher.

2.2.2. 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

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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 extract and all the fractions was lower than against A431 cells. As an example, the extract inhibited the viability of Jurkat cells with an IC_{50} of 10.9 μg/mL after a 72h treatment. The fraction consisting of pure haemanthamine (A9) resulted the most toxic against Jurkat cells, but the IC_{50} value was higher than that recorded for 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} matching with that found in our experiments (14.9 μM) [43]. 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 illustrated in the Supplementary Information file 2 (SI2).

2.2.3. 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 concentrations (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 [9,40]. The antibacterial action reported by Kallawaya 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* [45], besides polyphenols, whose antibacterial action is widely recognized [46].

3. Materials and Methods

3.1. 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. A voucher specimen of these plants (BOLO0602041) was deposited in the Herbarium of 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/.

3.2. 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-*d6* and $CDCl_3$ were purchased from Eurisotop (Saclay, France).

3.3. Preparation of the alkaloid-enriched extract

The alkaloid enriched extract was prepared according to the protocol proposed by Renault et al. [47] slightly modified. 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

 doi:10.20944/preprints202305.0921.v1

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 [33]. 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.

3.4. Centrifugal Partition Chromatography

The centrifugal partition chromatography (CPC) was carried out on 1 g of the bulb extract as reported by Lianza et al. [17]. Fraction 12 was further fractioned using a device adapted to its low mass (99.0 mg), the FCPC-A200 column of 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, Allschwil, Switzerland). The biphasic solvent system was the same as the one for extract fractionation, MtBE: CH_3CN: H_2O, in 5:2:3 (v/v) proportion. The concentration of retainer, H_2SO_4 1.5 mM, and of displacer, TEA 2 mM, were adapted to the small sample mass.

3.5. UPLC-HRMS

The Ultra Performance Liquid Chromatography coupled with High Resolution Mass Spectrometry (UPLC-HRMS) analysis were performed as already reported by Lianza et al. [17].

3.6. NMR

NMR analyses for spectra recording in DMSO-*d6* were performed according to Lianza et al. [17]. For the analysis in $CDCl_3$, the central resonance (septet) was set at δ_C 77.16 for ^{13}C NMR spectrum referencing and at δ_H 7.26 for ^{1}H NMR spectrum referencing.

3.7. Cytotoxic activity

3.7.1. 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.

3.7.2. 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 the 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 or 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 suspension Jurkat cell and a metabolic assay for the adherent A431 cells, using 4-methylumbelliferyl heptanoate (MUH, Merck, Darmstadt, Germany). Fluorescence was measured with the Guava EasyCyte 6-2L flow cytometer (Merck) or the 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) versus normalized response].

3.7.3. Statistical analysis

 doi:10.20944/preprints202305.0921.v1

The results are shown as the means ± SEM of at least two different experiments. Significant differences among treatments were assessed by two-way analysis of variance (ANOVA), 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.

3.8. Preparation of the extracts for antibacterial activity

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

3.8.1. Bacterial strains and antibacterial assay

Staphylococcus aureus ATCC 25293, *Staphylococcus epidermidis* (ATCC 12228), *Streptococcus pyogenes* (ATCC 19615) were selected as representative strains to test the antibacterial properties of plant extracts by using a standardized microdilution broth method 96-well plate [48] and 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 CFU (colony forming units)/mL, and subsequently diluted 1:200 in Brain Heart Infusion Broth (Sigma-Aldrich) and incubated with the extracts 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 the Optical Density at 630 nm (Multiskan Ascent microplate reader, Thermo Fisher Scientific Inc., Waltham, USA). Percentage values of bacterial growths were determined as relative to the positive control.

4. Conclusions

S. miniatum bulbs proved to be a source of pretazettine and haemanthamine, two Amaryllidaceae alkaloids endowed with potent cytotoxic activities, thus supporting the anticancer ethnobotanical use. Conversely, the alleged antibacterial properties were not ascertained, further study could be conducted on the analysis of non-alkaloid compounds produced by this species. Phytochemical characterization by dereplication allowed the rapid identification of twelve alkaloids, eleven of which were already know. The two artefacts identified in this study, namely tazettine and 6-dehydroxy-6-acetamido-nerinine, were produced by the use of NH_4OH during the extraction process, pointing out the needs of alternative methods for such a plant matrix.

Author Contributions: Conceptualization. M.L., J.M.N. and F.P.; methodology. M.L., J.M.N., J.H.R. and F.P; software. J.M.N.; validation. M.L, J.M.N; formal analysis. C.F., M.L., J.M.N, E.T.; investigation. M.L., G.G., E.T., F.B.; data curation. F.P, M.L, J.M.N., E.T., C.F., F.B.; writing—original draft preparation. M.L., F.P.; writing—review and editing. J.M.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The data presented in this study are openly available at zenodo.org from https://doi.org/10.5281/zenodo.4574016 .

Conflicts of Interest: The authors declare no conflict of interest.

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
UPLC Ultra performance liquid chromatography

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