Study of the inhibitory (toxic) effect of the alkaloids from chosen plants of Amaryllidaceae family on some human enzymatic systems (in vitro study) II.

Department of Pharmaceutical Botany
The Declaration

I declare that the material contained within this Thesis is original and contains no material which has been accepted for a degree or diploma by either the University or any other institution, except by way of background information and duly acknowledged in the Thesis. To the best of my knowledge and belief this Thesis contains no material previously published or written by another person except where due acknowledgement is made in the text of the Thesis.

Hradec Králové, August 2018

Mgr. Nina Vaněčková
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1 INTRODUCTION

Not only plants, but their metabolites as well, are parts of daily human life. They are used as a food source and have also been utilised for their medicinal or toxic effects for a very long time\(^1\). The monitoring of plant toxicity is of great importance nowadays. The purity and safety of a food chain is dependent on it because its pollution can cause some serious changes to the health of us all.

Although it does not look like it, natural remedies play an irreplaceable part in therapy. The growing number of articles about medicinal drugs in the European Pharmacopoeia and texts which declare medicinal plants as a source of active substances suitable for more research into their potential for drugs is undoubted proof of this.

There is a large group of secondary metabolites extracted from plants which have played an important role in medicine (e.g. morphine, thebaine, paclitaxel, flavonolignans of *Silybum marianum*, alkaloids of *Catharanthus roseus*, *Claviceps purpurea*, *Galanthus nivalis*, etc.)

A wide range of biological activities of secondary metabolites is tightly linked with the huge amount of chemical structures. One of the most interesting groups within the secondary metabolites is alkaloids. These are organic compounds which contain at least one nitrogen atom. They are synthesized from either amino acids or from intermediates which are formed during the biosynthesis of terpenoids, steroids, some acids and purines. The true importance of alkaloids has not been clarified yet but the most possible role is the protection of the plant against pests and their use in biosynthesis\(^2\).

Amaryllidaceae alkaloids are one of the most important groups of alkaloids. The amino acid thyrosine is their biosynthetic precursor. For this reason, they are classified among the isoquinoline alkaloids, although this class of alkaloids is produced by a wider range of plant families (e.g. Fumariaceae, Papaveraceae, Berberidaceae, Rutaceae, etc.). However, the Amaryllidaceae alkaloids are exclusively produced by plants of that family. So far, more than 500 alkaloids have been isolated and identified from the Amaryllidaceae family and series of them have been tested for a variety of biological activities. The alkaloids of the Amaryllidaceae family are significant and
biologically active, e.g. they are capable of the inhibition of human cholinesterase, and have apoptosis-inducing anticancer activity\textsuperscript{3,4}. It is interesting that biological activity is often connected with certain structural type.

These compounds are known these days especially thanks to galanthamine, which is used clinically under the commercial name Reminyl\textsuperscript{®} as a selective reversible inhibitor of acetylcholinesterase in the symptomatic treatment of Alzheimer’s disease. This disease is clinically manifested as dementia which is one of the main causes of death these days. However, the isolated alkaloids should not be studied only for their effect on the activity on human cholinesterases; the therapy of AD has to have a complex pharmacotherapeutical approach. So, the isolated alkaloids are/will be tested for their biological effect which can bring some new connections in affecting the neurodegenerative processes, e.g. the inhibition of prolyl oligopeptidase, GSK-3β.

This dissertation deals with the isolation, identification and biological activity of the alkaloids isolated from the fresh bulbs of \textit{Nerine bowdenii} Watson. This plant was chosen for phytochemical study on the basis of a GC/MS screening study performed on selected plants from the Amaryllidaceae family\textsuperscript{5,6,7}. It was intended to choose a plant which would be a source of the widest spectra of alkaloids, alkaloids not yet described, and which could be isolated in amounts which would allow us to perform the widest range of biological tests. \textit{Nerine bowdenii} has showed the most promising biological activity and the widest spectrum of alkaloids.
2 AIM OF THE DISSERTATION

The aim of this dissertation was the isolation and identification of the alkaloids from the concentrated alkaloidal extract prepared from the fresh bulbs of *Nerine bowdenii* Watson (family Amaryllidaceae) and their screening for biological activities connected to Alzheimer’s disease and oncological diseases.

Partial aims:

- detailed research of the literature mentioning alkaloids from the genus *Nerine* and their biological activity
- isolation of the wide spectrum of alkaloids from the bulb extract of *Nerine bowdenii* Watson
- determination of their structures based on their physical and chemical properties (NMR, MS, optical rotation, etc.)
- determination of the biological activities of the isolated alkaloids (inhibitory activity against human cholinesterases, prolyl-oligopeptidase, cytotoxic activity and others)
- selection of the most active alkaloids for further detailed biological studies, or the preparation of derivatives for structure-activity relationship studies (SAR study)
# 3 ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BuChE</td>
<td>Butyrylcholinesterase</td>
</tr>
<tr>
<td>CC</td>
<td>Column chromatography</td>
</tr>
<tr>
<td>ChEs</td>
<td>Cholinesterases</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P</td>
</tr>
<tr>
<td>ČL</td>
<td>Czech Pharmacopoeia</td>
</tr>
<tr>
<td>ČSN</td>
<td>Czech technical standards</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>f.a.</td>
<td>for analysis</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glycogen synthase kinase-3β</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Median lethal dose</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrum/spectrometry</td>
</tr>
<tr>
<td>N4OMT</td>
<td>Norbelladine 4'-O-methyltransferase</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>p.</td>
<td>pure</td>
</tr>
<tr>
<td>PAL</td>
<td>Phenylalanine ammonia lyase</td>
</tr>
<tr>
<td>PAMPA</td>
<td>Parallel artificial permeation assay</td>
</tr>
<tr>
<td>POP</td>
<td>Prolyl oligopeptidase</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-activity relationship</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>sp.</td>
<td>species</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>UHPLC</td>
<td>Ultra high-performance liquid chromatography</td>
</tr>
</tbody>
</table>
4 THEORETICAL PART

4.1 Amaryllidaceae family: history and traditional use

Species belonging to the Amaryllidaceae family grow wild in several countries\(^8\). They are widely distributed in the tropics and warm temperature regions of the world\(^9\). Distinct geographical locations are dominant: Andean South America, the Mediterranean basin and southern Africa\(^10\). The family includes about 1100 species of monocotyledonous perennial bulbaceous plants, which are classified into 75 genera\(^11\). About one third of these species grow in South Africa and are largely utilized in the traditional medicinal practices of the indigenous people\(^10\). We can also find representatives of 3 genera of the Amaryllidaceae family growing wild in the Czech Republic: daffodil (Narcissus, L.), snowflake (Leucojum, L.) and snowdrop (Galanthus, L.).

These plants are or were used in folk medicine. The first documented use of plants of the Amaryllidaceae family is dated in the era of the “father of medicine” Hippocrates of Kos (460-370 BC) who used an extract from Narcissus for the treatment of cancer in the area of the uterus\(^12\). His followers continued in the usage of Narcissus extracts (in particular extracts from Narcissus pseudonarcissus L. and Narcissus tazetta L.) for the treatment of cancer. The use of the extracts from Narcissus spread to China, North Africa, Central America and Arabian medicine in the Middle Ages\(^12\). Table 1 lists the use of the chosen plants in folk medicine.
Table 1. The use of plants from the Amaryllidaceae family in folk medicine

<table>
<thead>
<tr>
<th>Plant</th>
<th>Use</th>
<th>Country</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Amocharis coranica</em> Herb.</td>
<td>mental disorders, hysteria</td>
<td>South Africa</td>
<td>13</td>
</tr>
<tr>
<td><em>Boophane disticha</em> Herb.</td>
<td>sedative, anxiolytic</td>
<td>South Africa</td>
<td>14, 15</td>
</tr>
<tr>
<td><em>Boophane haemanthoides</em> F.M.Leight</td>
<td>asthma</td>
<td>South Africa</td>
<td>16, 17</td>
</tr>
<tr>
<td><em>Brunsvigia littoralis</em> R.A. Dyer</td>
<td>infertility</td>
<td>South Africa</td>
<td>18</td>
</tr>
<tr>
<td><em>Clivia miniata</em> Regel</td>
<td>infertility</td>
<td>South Africa</td>
<td>19</td>
</tr>
<tr>
<td><em>Crinum amabile</em> Donn</td>
<td>emetic, rheumatism, earache</td>
<td>Vietnam</td>
<td>20, 21</td>
</tr>
<tr>
<td><em>Crinum asiaticum</em> L.</td>
<td>tumors (abdomen)</td>
<td>Zaire, Indochina</td>
<td>20</td>
</tr>
<tr>
<td><em>Crinum bulbispermum</em> Milne-Redh. &amp; Schweick.</td>
<td>malaria, earache</td>
<td>South Africa</td>
<td>22, 23, 20</td>
</tr>
<tr>
<td><em>Crinum latifolium</em> L.</td>
<td>prostate carcinoma</td>
<td>Vietnam</td>
<td>24</td>
</tr>
<tr>
<td><em>Crinum L.</em></td>
<td>tumors, leprosy, cold, stomachache</td>
<td>South Africa, New Guinea</td>
<td>20</td>
</tr>
</tbody>
</table>

The biological activity of plants is often connected with the content of some specific type of secondary metabolites. In the case of the family Amaryllidaceae, the isoquinoline alkaloids, called Amaryllidaceae alkaloids, are the most significant secondary metabolites. Amaryllidaceae plants have been demonstrated to be a good source of unique alkaloids with a large biologically active spectrum and grouped in 12-ring-types. The uniqueness of these alkaloid structures provided a viable platform for the phytochemical-based drug discovery.

Investigation of the Amaryllidaceae alkaloids began in 1877 with the isolation of lycorine from *Narcisus pseudonarcissus*. Since then, the interest around this group of naturally occurring compounds increased because of the wide range of their biological properties displayed, including antitumor, antiviral, antibacterial, antifungal, antimalarial, analgesic and cytotoxic activities. In addition to these potential medicinal properties, Amaryllidaceae plants are known to be poisonous and these toxic effects have to be taken into account. To this day, some hundreds of these compounds have been isolated, identified and classified into several structural types. These alkaloids are included in the isoquinoline alkaloid group, especially due to their biosynthetic origin.

The main medicinal application of the Amaryllidaceae alkaloids is represented by galanthamine. Galanthamine is a selective acetylcholinesterase (AChE) inhibitor and it
was launched onto the market in 2000 as a hydrobromide salt (Reminyl®) for the treatment of Alzheimer’s disease, a progressive neurodegenerative disorder. In contrast with the other used AChE inhibitors, galanthamine also has the ability to allosterically modulate the nicotine receptors. It stops the desensitization of these receptors which increases the efficiency of the used medicines (agonists of the nicotine receptors), which can help to improve the symptomatology of the disease.

Although several chemical syntheses of galanthamine have been accomplished, plant extract still remained the main source for the pharmaceutical industry. In addition, a biotechnological process also provides a significant alternative approach for producing this valuable natural product. Originally, galanthamine was isolated from the bulbs of the green snowdrop or Woronow’s snowdrop (*Galanthus woronowii* Losinsk.). Galanthamine is present in practically all the genera of the Amaryllidaceae family; e.g. *Leucojum* L., *Lycoris* Herb., *Narcissus* L., *Zephyranthes* Herb., etc. Although the Amaryllidaceae is known especially for its content of this unique kind of alkaloids, various flavonoids, including flavones, chalcones and chromones, have been detected in the extracts.

### 4.2 Biosynthesis of Amaryllidaceae alkaloids: Norbelladine pathway

The Amaryllidaceae alkaloids are largely restricted to the family Amaryllidaceae, specifically the subfamily Amaryllidoideae. Some noteworthy exceptions are the collection of alkaloids that have been found in the genus *Hosta*, which is in the order Asparagales, along with Amaryllidaceae.

There are still new alkaloids being discovered in this group and even novel carbon skeletons with great potential to contribute to the list of known biologically active compounds. A diversity of carbon skeletons is known for this group of alkaloids including belladine, galanthamine, crinine, lycorine, galanthindole, homolycorine, galasine, montanine, cripowelline, cherylline, buflavine, plicamine, tazettine, graciline, augustamine, pancratistatine, gracilamine, etc. (more than 18 structural types of alkaloids) (Fig. 1).
Fig. 1. Structures of selected Amaryllidaceae alkaloids
The core biosynthetic pathway of the Amaryllidaceae alkaloids consists of the reactions required to produce 3,4-dihydroxybenzaldehyde and tyramine, the condensation and reduction of these precursors to norbelladine, and the subsequent methylation of norbelladine to 4′-O-methylnorbelladine (Fig. 2). Phenylalanine and tyrosine were shown to be precursors for haemanthamine by incorporation of [3-C\textsuperscript{14}]phenylalanine and [3-C\textsuperscript{14}]tyramine into haemanthamine in \textit{Nerine bowdenii}\textsuperscript{37}. The degradation experiments on haemanthamine generated from radiolabelled tyramine were used to demonstrate the placement of the labelled carbons on positions 11 and 12 in experiments with [2-C\textsuperscript{14}]tyrosine in \textit{Sprekelia formosissima} and [1-C\textsuperscript{14}]tyrosine in \textit{Narcissus cv. 'Twink'} daffodil\textsuperscript{38,39}. [3-C\textsuperscript{14}]Tyramine has also been documented to incorporate into haemanthamine, haemanthidine and 6-hydroxycrinamine in \textit{Haemanthus natalensis} bulbs\textsuperscript{40}.

The pathway for the conversion of phenylalanine to 3,4-dihydroxybenzaldehyde is shown in the following sequence: phenylalanine, \textit{trans}-cinnamic acid, 4-hydroxycinnamic acid, 3,4-dihydroxycinnamic acid or 4-hydroxybenzaldehyde, and 3,4-dihydroxybenzaldehyde\textsuperscript{41} (Fig. 2).
Fig. 2. The pathway for phenylalanine's conversion and the synthesis of 4'-O-methylnorbelladine
4'-O-Methylnorbelladine has been shown to be a precursor of all the primary alkaloid skeletons including crinine (crinine), haemanthamine (vittatine, 11-hydroxyvittatine), galanthamine (galanthamine, N-demethylgalanthamine and N-demethylnarwedine) and lycorine (lycorine, norpluviine and galanthine). 4'-O-Methylnorbelladine has long been considered the direct substrate for creation of the para-para' and ortho-para' carbon skeletons and it has also been established as the direct precursor of the para-ortho' skeleton as well. This universal requirement in all phenol-phenol coupling branches for 4'-O-methylnorbelladine makes it the last common intermediate before a three way split in the Amaryllidaceae biosynthetic pathway. The three common divisions at 4'-O-methylnorbelladine are the para-para' coupling that leads to the crinine and vittatine enantiomeric series, the ortho-para' phenol coupling that is elaborated into the classic alkaloid lycorine and the para-ortho' coupling that is elaborated into the most widely used Amaryllidaceae alkaloid galanthamine. Most of the other Amaryllidaceae alkaloid carbon skeletons are thought to be derivatives of these four skeletons; e.g. the pancratistatin e and tazettine carbon skeletons derived from the haemanthamine skeleton and the homolycorine skeleton derived from the lycorine skeleton. The belladine-type alkaloids are thought to originate by the simple methylation of norbelladine, though the order of methylations is not determined (Table 2).

Table 2. Structural type of alkaloids from the Amaryllidaceae family, their main representatives and the overview of the genera from which they were isolated

<table>
<thead>
<tr>
<th>Structural types</th>
<th>Main representative</th>
<th>Genus</th>
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<tbody>
<tr>
<td>belladine</td>
<td></td>
<td><em>Crinum</em>&lt;sup&gt;49,50,51&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td><em>Galanthus</em>&lt;sup&gt;52&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Chlidanthus</em>&lt;sup&gt;53,54&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Nerine</em>&lt;sup&gt;7,55&lt;/sup&gt;</td>
</tr>
<tr>
<td>Name</td>
<td>Chemical Structure</td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>------------------------</td>
<td></td>
</tr>
<tr>
<td>α-crinine</td>
<td><img src="image1" alt="Chemical Structure" /></td>
<td></td>
</tr>
<tr>
<td>β-crinine</td>
<td><img src="image2" alt="Chemical Structure" /></td>
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<tr>
<td>Crinine</td>
<td><img src="image3" alt="Chemical Structure" /></td>
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<tr>
<td>Haemanthamine</td>
<td><img src="image4" alt="Chemical Structure" /></td>
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<tr>
<td>Galanthamine</td>
<td><img src="image5" alt="Chemical Structure" /></td>
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<td>Lycorine</td>
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<tr>
<td>Galanthine</td>
<td><img src="image7" alt="Chemical Structure" /></td>
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<tr>
<td>Lycoris</td>
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<tr>
<td>Narcissus</td>
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<tr>
<td>Zephyranthes</td>
<td><img src="image10" alt="Chemical Structure" /></td>
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</tbody>
</table>
galanthindole

\[
\text{galanthindole} \quad \text{Galanthus}^{61,72}
\]

homolycorine

\[
\text{homolycorine} \quad \text{Clivia}^{57}, \text{Galanthus}^{61}, \text{Hippeastrum}^{62,63}, \text{Leucojum}^{69}, \text{Lycoris}^{65,66,67}, \text{Narissus}^{7,70}
\]

galasine

\[
\text{galasine} \quad \text{Galanthus}^{51}
\]

montanine

\[
\text{montanine} \quad \text{Hippeastrum}^{62,63}, \text{Lycoris}^{64}, \text{Pancratium}^{73}, \text{Scadoxus}^{5}
\]

cripowelline A

\[
\text{R1, R2 = -CH}_2\text{OCH}_3
\]

cripowelline B

\[
\text{R1 = R2 = CH}_3
\]

\[\text{cripowelline} \quad \text{Crinum}^{74}\]
cherylline

buflavine

plicamine

tazettine

graciline

Crinum$^{75}$

Narcissus$^{7,76}$

Boophane$^{77}$

Galanthus$^{78}$

Clivia$^{57}$

Eucharis$^{59}$

Galanthus$^{60,78}$

Hippeastrum$^{62,79}$

Chlidanthus$^{54,53}$

Leucojum$^{69}$

Zephyranthes$^{68}$

Galanthus$^{80}$
augustamine

pancratistatin

gracilamine

mesembrine

mesebrane

joubertiamine

skeletine A4

Crinum

Boophane

Haemanthus

Hymenocallis

Narcissus

Pancratium

Zephyranthes

Galanthus

Narcissus

Narcissus
4.3 Biological activity of the Amaryllidaceae alkaloids

As mentioned before, the Amaryllidaceae alkaloids have shown a wide range of bioactivity, including antiviral, antibacterial, antitumor, antifungal, antimalarial, analgesic and cytotoxic activity (Table 3).

Table 3. Biological activity of selected Amaryllidaceae alkaloids

<table>
<thead>
<tr>
<th>Alkaloids</th>
<th>Source</th>
<th>Biological activities</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lycorine</td>
<td><em>Sternbergia lutea</em> Ker Gawl</td>
<td>inhibition of ascorbic acid biosynthesis, anticytokine, anti-fungal, phytotoxic, anticancer, antiinflammatory</td>
<td>3, 90, 91, 92, 93, 94, 95, 96, 97</td>
</tr>
<tr>
<td>Amarbellisine</td>
<td><em>Amaryllis belladonna</em> L.</td>
<td>antibacterial, antifungal</td>
<td>93</td>
</tr>
<tr>
<td>Narciclasisine</td>
<td><em>Pancratium maritinum</em> L.</td>
<td>antimitotic, anticancer, micotoxic</td>
<td>98, 99, 100</td>
</tr>
<tr>
<td>Haemanthamine</td>
<td><em>Haemanthus</em> sp.</td>
<td>anticancer</td>
<td>93, 97, 101</td>
</tr>
<tr>
<td>Haemanthidine</td>
<td><em>Haemanthus</em> sp.</td>
<td>anticancer</td>
<td>93, 97, 101</td>
</tr>
<tr>
<td>11-Hydroxyvittatine</td>
<td><em>A. belladonna</em> L.</td>
<td>antibacterial</td>
<td>95</td>
</tr>
<tr>
<td>Vittatine</td>
<td><em>A. belladonna</em> L.</td>
<td>antibacterial</td>
<td>95</td>
</tr>
<tr>
<td>Bulbispermine</td>
<td><em>Crinum bulbispernum</em></td>
<td>anticancer, antiinflammatory</td>
<td>96, 97, 102</td>
</tr>
<tr>
<td>Crinsarnine</td>
<td><em>Nerine sarniensis</em></td>
<td>insecticide</td>
<td>103</td>
</tr>
<tr>
<td>Sarniensine</td>
<td><em>N. sarniensis</em></td>
<td>insecticide</td>
<td>104</td>
</tr>
<tr>
<td>Pretazettine</td>
<td><em>Narcissus tazetta</em> L.</td>
<td>anticancer</td>
<td>12</td>
</tr>
<tr>
<td>Tazettine</td>
<td><em>N. tazetta</em> L.</td>
<td>antimalarial</td>
<td>12</td>
</tr>
<tr>
<td>Jonquailine</td>
<td><em>Narcissus jonquilla</em> Quail</td>
<td>anticancer</td>
<td>105</td>
</tr>
</tbody>
</table>

Nowadays, the ability to inhibit AChE is the most significant activity of these alkaloids. This inhibition is mainly used in the treatment of AD\textsuperscript{106}. AD is a major neurodegenerative disease which is clinically manifested by a dementia. The cause of this disease is not known, therefore the pharmacotherapy cannot be as effective as in the case of other positively clarified diseases\textsuperscript{107}.

From the beginning, AD is characterized by a defect in the central acetylcholinesterase system. As a result of this defect, the production and release of acetylcholine is significantly lowered from the presynaptic cell. The acetylcholine is degraded by acetylcholinesterases in the synaptic cleft. There are two forms of this enzyme which are occurring in the brain: G4 which is the major form of the enzyme and G1, the minor form, whose content increases with AD\textsuperscript{108}. The enzyme
butyrylcholinesterase also plays a part in the degradation of acetylcholine. We can find BuChE in the neurons and glial cells, as well as in the neuritic plaques and tangles of patients with AD. The activity of this enzyme increases with age with AD patients while the activity of AChE is not changed or, in fact, is lowered\textsuperscript{109,110}. The central cholinesterase inhibitors are the most important and most effective drugs. They are used in the therapy of the early and intermediate states of dementia. The application of these drugs leads to the slowdown of the symptoms of the dementia and the delay of the advanced stages of the dementia where the patients are not self-sufficient and are dependent on the help of others\textsuperscript{107}.

One of the most important substances with the ability to inhibit AChE is an alkaloid isolated from the family Amaryllidaceae, specifically galanthamine (IC\textsubscript{50} = 1.07 µM). It was isolated first from the bulbs of \textit{Galanthus woronowii} Losinsk. and later from other plants of the family Amaryllidaceae\textsuperscript{111}. From the launch of galanthamine into clinical practise, other Amaryllidaceae alkaloids received attention as potential AChE inhibitors. Besides galanthamine, other alkaloids were isolated from the family with the same or even higher inhibitory activity against AChE. Most of these were galanthamine or lycorine type alkaloids. One such was sanguinine, which was isolated from \textit{Galanthus elwesii} Hook\textsuperscript{112} and \textit{Lycoris sanguinea} Maxim\textsuperscript{113}. Sanguinine has a similar structure to galanthamine, the only difference being that sanguinine has a methoxy group instead of a hydroxy group. This small change in the structure results in a ten times increase of the inhibitory activity compared with galanthamine\textsuperscript{114}. The same effect was described with the allyl group on the N-atom, such as \textit{N}-allylnorgalanthamine and \textit{N}-(14-methylallylnorgalanthamine)\textsuperscript{115}. On the other hand, the elimination of the methyl group on the \textit{N}-atom, as it is with epinorgalanthamine, leads to a ten times decrease in the inhibitory activity. Hydrogenation of the double bound in the cyclohexene leads to the complete loss of the inhibitory effect (lycoramine, epinorlycoramine)\textsuperscript{114}. From the point of view of the inhibitory effect, chlidanthine was considered as a very interesting substance. It is a positional isomer of galanthamine; there is a substitution of the hydroxy and methoxy group. However, all of the performed studies have shown that the inhibitory activity of this alkaloid is significantly lower than that of galanthamine\textsuperscript{54,116}. The relationship between the structure of the alkaloid and its inhibitory activity against BuChE is not possible to define due to the small number of
tested substances. In fact, almost all of the studies dealing with the BuChE inhibitory activity of the Amaryllidaceae alkaloids were performed in the laboratory of our department\textsuperscript{68,54}. (Table 4).

The inhibition of prolyl oligopeptidase (POP) is another interesting approach for the supportive treatment of AD. POP is a cytosolic serine peptidase which cleaves small peptides containing the amino acid proline. The substrates of these enzymes are e.g. substance P, vasopressin and some peptides. These substrates are operating as modulators of the cognitive function and their degradation can accelerate the process of ageing or play a role in neurodegenerative diseases\textsuperscript{117}.

Table 4. The inhibitory activity of some chosen alkaloids isolated from the family Amaryllidaceae against AChE and BuChE

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>Structural type</th>
<th>IC\textsubscript{50} AChE (µM)</th>
<th>IC\textsubscript{50} BuChE (µM)</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>sanguinine</td>
<td>galanthamine</td>
<td>0.10 ± 0.01</td>
<td>not measured</td>
<td>114</td>
</tr>
<tr>
<td>galanthamine</td>
<td>galanthamine</td>
<td>1.07 ± 0.18</td>
<td>43.3 ± 1.3</td>
<td>68,114</td>
</tr>
<tr>
<td>11-hydroxygalanthamine</td>
<td>galanthamine</td>
<td>1.61 ± 0.21</td>
<td>not measured</td>
<td>114</td>
</tr>
<tr>
<td>N-allylnorgalanthamine</td>
<td>galanthamine</td>
<td>0.18</td>
<td>not measured</td>
<td>115</td>
</tr>
<tr>
<td>(N)-(14-methylallyl)norgalanthamine</td>
<td>galanthamine</td>
<td>0.16</td>
<td>not measured</td>
<td>115</td>
</tr>
<tr>
<td>chlidantine</td>
<td>galanthamine</td>
<td>147 ± 6</td>
<td>422 ± 15</td>
<td>54</td>
</tr>
<tr>
<td>epinorlycoramine</td>
<td>galanthamine</td>
<td>&gt;500</td>
<td>not measured</td>
<td>114</td>
</tr>
<tr>
<td>lycoramine</td>
<td>galanthamine</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>68,114</td>
</tr>
<tr>
<td>oxoassoanine</td>
<td>lycorine</td>
<td>47.21 ± 1.13</td>
<td>not measured</td>
<td>114</td>
</tr>
<tr>
<td>assoanine</td>
<td>lycorine</td>
<td>3.87 ± 0.24</td>
<td>not measured</td>
<td>114</td>
</tr>
<tr>
<td>lycorine</td>
<td>lycorine</td>
<td>&gt;500</td>
<td>not active</td>
<td>54,68,114</td>
</tr>
<tr>
<td>1-O-acetyllycorine</td>
<td>lycorine</td>
<td>0.96 ± 0.04</td>
<td>not measured</td>
<td>118</td>
</tr>
<tr>
<td>1,2-di-O-acetyllycorine</td>
<td>lycorine</td>
<td>211 ± 10</td>
<td>not measured</td>
<td>118</td>
</tr>
<tr>
<td>2-O-acetyllycorine</td>
<td>lycorine</td>
<td>&gt;500</td>
<td>not measured</td>
<td>118</td>
</tr>
<tr>
<td>8-O-demethylmaritidine</td>
<td>crinine</td>
<td>28.0 ± 0.9</td>
<td>not active</td>
<td>68</td>
</tr>
<tr>
<td>undulatine</td>
<td>crinine</td>
<td>23.0 ± 1.0</td>
<td>not active</td>
<td>54</td>
</tr>
</tbody>
</table>

Antitumor activity is another very interesting biological activity of the Amaryllidaceae alkaloids. This activity is based on the induction of apoptosis. The selectivity of these alkaloids against the cancer cells and their relatively low toxicity for
the healthy and resting cells are the biggest advantages of these alkaloids. The most active substances are from these three structural types: lycorine, crinine and pancratistatine.

Oncological diseases are one of the leading causes of death in the developed countries and an increase in its prevalence seems to be inevitable. We have witnessed a tremendous advance in the understanding and treatment of cancers during the last decades. Nevertheless, the oncological patients still die due to a resistance of cancer cells to the therapy. These resistant cells drive the growth of the tumors and contribute to the dissemination of the malignant cells into the vital organs. The common mechanisms responsible for the cancer cells represent up-regulation of DNA damage sensing and the repair capacity of the cells, induction of cell cycle arrest allowing time for the DNA damage repair and defects in the apoptosis signalling machinery.

Cancer of the lungs, breast, colon, stomach and prostate are the most frequent. Most of the patients die due to the expansion of the metastasis into the vital organs. The standard treatment consists of surgical interventions, radiation cycles and chemotherapy. The chemotherapy can also damage the healthy tissue which can cause a lot of side effects. A serious complication of the treatment is the resistance of the cancer cells to the used chemotherapeutics. In some cases, the resistance can extend to chemotherapeutics which differ structurally and also have different mechanisms of action. This is referred to as multiple drug resistance.

It is well known that many proteins control the cell death machinery. An example is protein p53, a transcription factor regulating downstream genes important in cell cycle arrest, DNA damage repair and cell death. The mutations of the tumor suppressor p53 can be frequently observed in malignant colorectal tumors. The mutations or deficiency of p53 are thought to reflect the tumor aggressiveness and resistance, and to be indicative of a lower survival rate. The activation of the wild-type (functional) p53 stimulates transcription of the proapoptotic molecules and there is also evidence that the wild-type p53 can directly enter the mitochondria and stimulate cell death. Because the p53 mutants are highly expressed in cancer cells (in over 50% of the human cancers) and they are not present in normal healthy cells, target therapy against the p53 deficient cells may eliminate these cancer cells by cell death. Therefore, substantial efforts are
being invested into identifying and developing compounds that would be able to target the resistant forms of tumor cells while not damaging the healthy cells\textsuperscript{122,127}.

Among the various natural sources that have been investigated for constituents with the potential for use in cancer treatment, the plants of the Amaryllidaceae family have been particularly promising and fruitful\textsuperscript{128}.

Currently many Amaryllidaceae alkaloids have been reported to exhibit promising antitumor properties\textsuperscript{129,130}. Of these, lycorine\textsuperscript{131}, narciclasine\textsuperscript{132} and pancratistatine\textsuperscript{84,133,134,135} are frequently described as cytotoxic at micromolar concentrations.

4.3.1 Lycorine alkaloids subgroup

Lycorine

Lycorine belongs to the pyrrole[de]phenanthridine ring-type subgroup\textsuperscript{12}. It is the main alkaloid extracted from the different Amaryllidaceae genera. Its structure was first determined by Nagakawa \textit{et al.}, in 1956\textsuperscript{136,137}. The first study on the biological activity of lycorine, revealed its ability to inhibit ascorbic acid biosynthesis\textsuperscript{90}. The demand for its availability prompted the development of a new extraction method from the dried bulbs of \textit{Sternbergia lutea} Ker Gawl, the best source of lycorine\textsuperscript{138,139}. Several hemisynthetic derivatives of lycorine were prepared as a relatively large amount of lycorine was available. These derivatives were used together with some other natural Amaryllidaceae alkaloids to carry out SAR studies testing their ability to inhibit ascorbic acid biosynthesis in potato tuber\textsuperscript{140,141}. Studies on the biodegradation of lycorine were also carried out using \textit{Pseudomonas} strains isolated from \textit{S. lutea} rhizosphere. The main metabolite obtained through bacterial transformation was identified as ungremine, while the other metabolites were anhydrolycorinium chloride and, probably, the dihydroderivative of ungremine, which was not isolated due to its instability\textsuperscript{142}.

The interest in lycorine and the Amaryllidaceae alkaloids was more recently renewed thanks to the discovery of their strong anticancer activity. Twenty-two lycorine-related compounds were investigated for their \textit{in vitro} antitumor activity using four cancer cell lines, displaying different levels of resistance to proapoptotic stimuli, and two cancer cell lines sensitive to proapoptotic stimuli. Lycorine appeared to be the
strongest anticancer compound in a single-digit micromolar range and showed a cytostatic rather than cytotoxic effect. Furthermore, lycorine displayed significant therapeutic benefit at nontoxic doses in mice bearing grafts of the B16F10 melanoma model. It appeared to be an excellent lead for the generation of compounds which are able to combat cancers naturally resistant to the proapoptotic stimuli, such as glioblastoma, melanoma, nonsmall-cell lung cancers and metastatic cancers.

Lycorine, amarbellisine, haemanthamine and haemanthidine are potentially useful chemical scaffolds to generate new compounds which are able to combat cancers associated with poor prognoses, especially those naturally resistant to apoptosis. Therefore, 32 analogues of lycorine were synthesized and evaluated for their in vitro antiproliferative activities in a panel of tumor cell lines including those exhibiting resistance to proapoptotic stimuli. Among them, 1,2-di-O-allyllycorine was identified as 100 times more potent than lycorine against a U373 human glioblastoma model, while some other synthetic analogues appeared to be promising for further in vivo studies.

Amarbellisine

Amarbellisine was isolated from the bulbs of Amaryllis belladonna L. (also named Hippeastrum equestre) cultivated in Egypt as an ornamental plant, together with the well-known alkaloids lycorine, pancraine, vittatine, 11-hydroxyvittatine and hippeastrine. The new alkaloid, amarbellisine, contains the pyrrolo[de]phenanthridine ring system. Amarbellisine, pancraine, 11-hydroxyvittatine and vittatine were tested for their antibacterial activity against Gram-positive Staphylococcus aureus and Gram-negative Escherichia coli. All of them showed antibacterial activity against S. aureus, while only amarbellisine and vittatine were active against E. coli and only pancraine was active against Pseudomonas aeruginosa. Finally, only lycorine, amarbellisine and hippeastrine showed antifungal activity against Candida albicans among all the isolated alkaloids.
4.3.2 Pancratistatine alkaloids subgroup

The alkaloids of this group are derived from phenanthridine.

Narciclasine

Narciclasine, an isocarbostyryl related to lycorine, also showed an interesting strong anticancer activity. For the first time, it was isolated from different species of Narcissus bulbs as a metabolite with antimitotic activity. Narciclasine, at ~1 μM \textit{in vitro} and ~10 mg/kg \textit{in vivo}, displayed marked proapoptotic and cytotoxic activity, as did pancratistatine, albeit also showing severe toxic side effects. However, at physiological doses, narciclasine is not cytotoxic, but cytostatic and displays marked \textit{in vivo} anticancer activity in experimental models of brain cancer without any toxic side effect. This activity involves the impairment of actin cytoskeleton organization. When narciclasine was chronically supplied at 1 mg/kg, it significantly increased the survival of immunodeficient mice orthotopically xenografted with highly invasive human glioblastomas and apoptosis-resistant brain metastases, thus appearing as a potentially promising agent for the treatment of primary brain cancers and various brain metastases. Narciclasine is bound to the ribosomal subunit 60S, where it can inhibit peptidyl transferase and therefore it prevents the formation of the peptide bond in the newly formed protein.

Pancratistatine

In some studies it was observed that pancratistatin can inhibit the growth of HeLa cells and is highly toxic against other types of leucemic cells. Recently, the high selectivity against cancer cells of pancratistatin was observed. The study was made \textit{in vitro} and \textit{in vivo}, on two types of colorectal cancer cells, p53-mutated HT-29 and p53-wild-type HCT compared with healthy fibroblasts of the column CCD-18Co. Pancratistatin caused cytotoxic induction of apoptosis, but only against the cancer cells. The apoptosis was caused by the decrease in the mitochondrial membrane potential without the detected creation of DNA-double strand breaks. In another study, the cytotoxic effect of pancratistatin was proven against two prostate cancer cell lines: LNCaP and DU145.
4.3.3 Crinine alkaloids subgroup

The *Crinum* alkaloids belong to the 5,10b-ethanophenanthridine ring-type subgroup\(^\text{12}\). Crinine alkaloids are considered as weak inhibitors of AChE. The stereochemistry of the 5,10b-ethano bridge has no effect on the activity of the substance. Based on the orientation of the 5,10b-ethano bridge, the alkaloids are divided into \(\alpha\)- and \(\beta\)-crinine types. Some literature divides these alkaloids into crinine and haemanthamine subtypes\(^\text{118}\).

Haemanthamine and haemanthidine

Haemanthamine, the most abundant Amaryllidaceae alkaloid, was first isolated from *Haemanthus* hybrids and successively from several different Amaryllidaceae genera such as *Crinum*, *Narcissus*, *Hymenocallis*, and *Zephyrantes*\(^\text{147}\). Later on, haemanthidine was also isolated from *S. lutea* as a minor alkaloid\(^\text{140,141}\). Haemanthamine and haemanthidine were also isolated together with ismine from *Sprekelia formosissima*\(^\text{148,149}\). Both haemanthamine and haemanthidine showed anticancer activity when tested against six cancer cell lines\(^\text{3}\).

Vittatine and 11-hydroxyvittatine

Vittatine was first isolated from *Hippeastrum vittatum*, *Nerine cornusca* and *Pancratium illyricum*\(^\text{150}\).

For the first time, 11-hydroxyvittatone was isolated from plants belonging to the *Sternbergia* genus and then from *Hippeastrum* hybrids\(^\text{151}\). Both of the alkaloids, vittatine and 11-hydroxyvittatine, were also isolated from *Amaryllis belladonna* and both showed antimicrobial activity\(^\text{93}\).

11-O-Acetylhaemanthamine and bujeine

11-O-acetylhaemanthamine and bujeine were isolated from the whole plants of *Narcissus bujei* together with the already known alkaloids: homolycorine, 8-O-demethylhomolycorine, masonine, lycorenine, O-methyllycorenine, O-methyloduline, crinamine, haemanthamine and tazettine\(^\text{152}\).

Bulbispermine

Bulbispermine is a very well-known alkaloid. It was isolated from *Crinum bulbispermum* for the first time\(^\text{22,153,154}\). *C. bulbispermum* is a plant in the Amaryllidaceae family which is native to South Africa, Lesotho and Swaziland, but it is
also naturalized in the Lesser Antilles, Honduras, Cuba and some parts of the USA. More recently, a significant amount of bulbispermine was isolated from *Zephyranthes robusta*\textsuperscript{102}, a species of the herbaceous flowering bulb commonly known as Brazilian copper lily. It is native to Brazil, Argentina and Uruguay, but is naturalized in Florida, Colombia and South Africa\textsuperscript{22,153,154}. As the anticancer activity of the crinine-type alkaloids remained substantially unexplored, bulbispermine, some of its new hemisynthetic derivatives and closely related alkaloids such as ambelline, buphanamine, buphanisine, haemanthamine and haemanthidine were assayed, \textit{in vitro}, against a panel of cancer cell lines with various levels of resistance to proapoptotic stimuli. Bulbispermine, haemanthamine and haemanthidine showed the most potent antiproliferative activities. Among the synthetic bulbispermine analogues, only the C1, C2-dicarbamate derivative showed strong growth inhibition properties. Bulbispermine also inhibits the proliferation of the glioblastome cells through cytostatic effects. This suggests that crinine-type alkaloids could be potentially useful drugs which could lead to the treatment of apoptosis-resistant cancers, such as glioblastoma\textsuperscript{102}.

\textbf{1-Epidemethylbowdensine}

1-Epidemethylbowdensine was isolated from the fresh leaves of *Crinum erburescens*, an Amaryllidaceae plant collected in Costa Rica, together with bowdensine, 1-epidemethoxybowdensine, crinamine, 11,12-dehydroanhyrolycorine, macronine, ismine and trisphaedrine\textsuperscript{155}.

Among all of the Amaryllidaceae alkaloids and the related isocarbostyryls, those belonging to the crinine type, characterized by the presence of the 5,10b-ethanophenanthridine ring system, have recently been shown to be effective for inhibition of the proliferation of cancer cells resistant to the various proapoptotic stimuli of the tumors with dismal prognoses which are refractory to the current chemotherapy, such as glioma, melanoma, nonsmall-cell lung, esophagal, head and neck cancers, among others\textsuperscript{156}. These results prompted the development of a straightforward biomimetic route for the compounds with a crinine skeleton. A collection of crinine analogues was prepared synthetically and evaluated against the cancer cells. Some of these synthetic derivatives showed single-digit micromolar activities and retained this activity against a variety of drug-resistant cancer cell cultures\textsuperscript{157}. 
4.3.4 Mesembrine alkaloids subgroup

Crinsarine, saniensine and saniensinol

The alkaloids of the mesembrine type are typical metabolites of Aizoaceae-Masembry-Anthemoiade. The first three Amaryllidaceae alkaloids belonging to the mesembrine type, isolated from Hymenocallis arenicola, Crinum oliganthum and Narcissus pallidulus, were beamisine, mesembrenol and mesembrenone, respectively. Later, egonine was obtained from Hippeastrum equestre. Mesembrine type alkaloids have been isolated from different species of Sceletium and reviewed in comparison with the Amaryllidaceae alkaloids by Jin.

Recently, some new mesembrine type alkaloids have been isolated from Nerine sarniensis. This Amaryllidaceae species, known as the Guernsey Lilly, is restricted to the Western Cape of South Africa. It has been widely studied by horticulturists, specifically for its aesthetic appeal. Together with the known alkaloids 1-O-acetyllycorine, bowdesine, lycorine, hippadine and tazettine, from the organic extract of N. sarniensis were isolated two new alkaloids belonging to the mesembrine group named sarniensinol and saniensine, and a new crinine type alkaloid named crinsarine.

In a preliminary study, the organic extract of N. sarniensis bulbs showed strong adulticidal and larvicidal activity against Aedes aegypti, which is the major vector of the arboviruses responsible for dengue and yellow fevers and Zika viral diseases, which are major threats to public health. There is no approved vaccine against Zika virus and the vaccine against dengue has only been registered recently.

Thus, all the alkaloids isolated from N. sarniensis were assayed against A. aegypti. None showed mortality against the 1st instar A. aegypti larvae at the tested concentrations.

In the adult topical bioassays, only crinsarine displayed strong adulticidal activity with an LD50 = 2.29 ± 0.049 μg/mosquitos.

As for the structure-activity relationship, the pretazettine scaffold in sarniensinol and saniensine and the crinine one in crinsarine and bowdesine proved to be important for their activity in the same way as the pyrrole[de]phenanthridine scaffold was for the activity of hippadine and 1-O-acetyllycorine. Among the pretazettine group, the opening of the B ring or the presence of a B ring lactone, as well as the trans-stereochemistry of...
the A/B ring junction appears to be important for the activity. In the crinine type of alkaloids, the substituent at C-2 seems to play a role in their activity\textsuperscript{103,161}.

### 4.3.5 Tazettine alkaloid subgroup

**Tazettine**

Tazettine is one of the most abundant alkaloids of this subgroup of Amaryllidaceae alkaloids\textsuperscript{163,164}. More recently, it has also been isolated from *Sprekelia formosissima* together with haemanthamine, haemanthidine and ismine, and from *Isnaene calithina* together with galanthamine, homolycorine, lycorine and nerinine\textsuperscript{165}. Tazettine exhibited antimalarial activity when tested against *Plasmodium falciparum*\textsuperscript{12}.

**Pretazettine**

Pretazettine was the main alkaloid isolated from *Ismene calithina*; it differs from tazettine in the substitutions at C-6a and at C-8\textsuperscript{165}. The physical and chemical properties recorded for pretazettine were in good agreement with those reported previously for isotazettine\textsuperscript{166}. The initial name isotazettine was not continued in use because it introduced a confusion with the existing references to isotazettine (criwelline), tazettinol and isotazettinol. These are all C-6a hydroxy derivatives of the [2]benzopyrano[3,4-c]indole nucleus, but they vary in stereochemistry. Pretazettine exhibited a potent growth-inhibitory activity against HeLa cells\textsuperscript{12}.

**Jonquailine**

Jonquailine was isolated from *Narcissus jonquailla* Quail, together with haemanthamine, lycorine and narciclasine\textsuperscript{167}. The initial biological evaluation of jonquailine revealed significant antiproliferative effects against glioblastoma, melanoma, uterine sarcoma and nonsmall-cell lung cancer cells displaying various forms of drug resistance, including resistance to apoptosis and multidrug resistance. Jonquailine was also found to synergize with paclitaxel in its antiproliferative action against drug-resistant lung cancer cells. The obtained results compared with the literature data also showed that the hydroxylation at C-8 is an important feature for the anticancer activity. However, it seems to be unaffected by either the stereochemistry or the acetalization of the lactol\textsuperscript{105}.
4.3.6 Galanthamine alkaloid subgroup

The alkaloids belonging to this subgroup have a dibenzofuran as a main constituent of their structure.

Galanthamine

Galanthamine is the most important representative of this subgroup. It differs from the other subgroups mainly by its biological activity. It was isolated for the first time from the bulbs of *Galanthus woronowii*. Later, it was isolated also from other species of Amaryllidaceae. The biological activity of galanthamine was observed by Bulgarian pharmacologists at the beginning of the 1950s\textsuperscript{168}. It is capable of the reversible competitive inhibition of acetylcholinesterase, which is used in the therapy of AD\textsuperscript{144}. It was observed that the therapy of this neurodegenerative disease by galanthamine leads to improvement in cognitive, behavioral and functional symptoms. The advantage of galanthamine is its higher selectivity to AChE than to BuChE\textsuperscript{168}. Galanthamine has no cytotoxic activity\textsuperscript{129}.

4.4 The genus Nerine

The genus *Nerine* Herbert (Amaryllidaceae) is a representative of the subfamily Amaryllidoideae, which is confined to the temperate regions of southern Africa\textsuperscript{169}. This genus, the second largest within the Amaryllidaceae with ca. 23 species, is an autumn-flowering, perennial, bulbous plant group, whose species inhabit areas with summer rainfall and cool, dry winters. Most *Nerine* species have been cultivated for their elegant flowers, presenting a source of innumerable horticultural hybrids\textsuperscript{169,170}. The exploitation of plants from this genus in the traditional medicinal practices of the indigenous people of southern Africa has been documented\textsuperscript{23}. In particular, the southern Sotho and Zulu tribes have used the decoctions from the bulbs in the treatment of coughs and colds, renal and hepatic conditions, to relieve back pain and as a remedy for infertility\textsuperscript{55}.

4.4.1 Nerine filifolia

This is an evergreen plant, with small bulbs and thin thread-like leaves. It flowers between September and November and occurs in the Eastern Cape, Transkei, Orange Free State, Swaziland and Mpumalanga. *Nerine filifolia* Baker (Fig. 5) was examined for its alkaloidal constituents. Following the usual extractive and chromatographic procedures, eight alkaloids, as well as phenol, were isolated. The known compounds
belladine, 11-\(O\)-acetylbelladine and undulatine were isolated as the main compounds in addition to the minor components – ambelline and 6\(\alpha\)-hydroxybuphandrine. \(N\)-demethylbelladine, 6\(\alpha\)-methoxybuphandrine and fililofline (the 11-\(O\)-nicotinyl analogue of ambelline) were reported for the first time as natural products\(^{55}\). A small quantity of galanthamine was detected in one phytochemical analysis of \textit{Nerine filifolia}\(^{171}\). In another study of the alkaloidal profile of \textit{N. filifolia}, seven compounds showed MS fragmentation patterns characteristic of Amaryllidaceae alkaloids, and six of them were identified as masonine, \(N\)-demethylmasonine, caranine, lycorine, crinine, acetylcaranine and \(O\)-methyloduline (Fig. 3). The alkaloidal pattern was dominated by homolycorine type alkaloids\(^7\).

![Structures of alkaloids isolated from \textit{Nerine filifolia}](image)

Fig. 3. Structures of alkaloids isolated from \textit{Nerine filifolia}
Fig. 3. Structures of alkaloids isolated from *Nerine filifolia* (continued)

### 4.4.2 *Nerine huttoniae*

*Nerine huttoniae* Schönland (Fig. 5) is a summer growing, evergreen species with large bulbs and sharp-shaped, shiny, dark green leaves. It occurs in the western part of Eastern Cape Province of South Africa where it grows in colonies near riverbanks or in seasonally damp depressions\(^{172,173}\). In the screening for CNS-active alkaloids from the family Amaryllidaceae, the alkaloidal extract from *Nerine huttoniae* showed inhibitory activity against acetylcholinesterase. Four alkaloids were isolated from this plant of which one was completely new: lycorine, tazettine, oxokrigenamine, 6-\textit{O}-methylkrigeine\(^{174}\) (Fig. 4).

Fig. 4. Structures of alkaloids isolated from *Nerine huttoniae*
Fig. 5. Chosen species from the genus *Nerine* Herbert; from the left: *Nerine filifolia*\textsuperscript{175}, *Nerine huttoniae*\textsuperscript{176}, *Nerine bowdenii*\textsuperscript{177}, *Nerine sariensis*\textsuperscript{178}, *Nerine filamentosa*\textsuperscript{179}, *Nerine undulata*\textsuperscript{180}

### 4.4.3 *Nerine filamentosa*

The alkaloid pattern of *N. filamentosa* (Fig. 5) was dominated by three crinine-type alkaloids: undulatine, buphanamine and ambelline, and one lycorine-type alkaloid, tentatively identified as acetylparkamine. Both parkamine and acetylparkamine (Fig. 6) were identified by comparing their MS with those of already known compounds of the same structural type (caranine, acetylcaranine, falcetine and acetylfalcetine) available in the NIST library\textsuperscript{7}. 

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4.4.4 Nerine undulata

Previous phytochemical investigation of *N. undulata* (Fig. 5) led to the isolation and identification of three alkaloids (lycorine, ambelline and undulatine)\(^{181}\). The alkaloid pattern of *N. undulata* in our study was dominated by two crinine-type alkaloids – undulatine, buphanamine and two unidentified alkaloids. Some other minor alkaloids were also identified – buphanidrine, ambelline, bowdesine, crinamidine and flexinine\(^{5}\) (Fig. 7).

4.4.5 Nerine sarniensis

*N. sarniensis* (Fig. 5) is considered as one of the most beautiful of all Nerines. It is also known as Guernsey Lily. This species is restricted to the Western Cape of South Africa\(^{160}\). The alkaloids from *N. sarniensis* remain largely unexplored. So far, tazettine, nerinine\(^{182}\), lycorine, 3-epimacronine and sarniensine have been isolated\(^{103}\) (Fig. 8).
Fig. 8. Structures of chosen alkaloids isolated from *Nerine sariensis*

### 4.4.6 *Nerine bowdenii*

*N. bowdenii* (Fig. 5) is an endemic Amaryllidaceae species native to KwaZulu-Natal, Drakensberg and Eastern Cape Provinces in South Africa and it is widely used as an ornamental flower. Previous phytochemical studies led to the isolation of about 25 Amaryllidaceae alkaloids. Our previous phytochemical study of the alkaloid extract of this species led to the detection of 22 compounds, 18 of which were identified by GC-MS – namely two isomers of buphanisine, crinine, belladine, caranine, acetylcaranine, *N*-demethylbelladine, acetylfalcatine, buphanidrine, buphanamine, crinamine, powelline, undulatine, 11-*O*-acetylambelline, ambelline, acetylnerbowdine, crinamidine, bowdesine, 11-*O*-acyt-1,2-epoxyambelline (Fig. 9).

Fig. 9. Structures of alkaloids isolated from *Nerine bowdenii*
5 EXPERIMENTAL PART

5.1 Materials and instruments used for the isolation of Amaryllidaceae alkaloids

5.1.1 Chemicals used for the isolation of alkaloids
- Anhydrous sodium carbonate p. (Ing. Švec – Penta, Praha)
- Bismuth subnitrate (Lachema, Brno) (BiNO₃(OH)₂ . BiO(OH))
- Diethylamine f.a. (Ing. Švec – Penta, Praha) (Et₂OH, DEA)
- Hydrochloric acid 35% f.a. (Ing. Švec – Penta, Praha) (HCl)
- Mercuric chloride f.a. (Fisher Scientific, Pardubice) (HgCl₂)
- Potassium iodide p. (Lach-Ner, Neratovice) (KI)
- Sodium hydroxide p. (Ing. Švec – Penta, Praha) (NaOH)
- Sulfuric acid 96% f.a. (Ing. Švec – Penta, Praha) (H₂SO₄)
- L- (+)-Tartaric acid f.a. (Balex, Pardubice) (C₄H₆O₆)
- Water solution of ammonia 22-25% f.a. (Ing. Švec – Penta, Praha) (NH₄OH)

5.1.2 Solvents used for the isolation and structural identification of Amaryllidaceae alkaloids
- Acetone f.a. (Ing. Švec – Penta, Praha) (Me₂CO)
- Chloroform p. (Ing. Švec – Penta, Praha) (CHCl₃)
- Cyclohexane p. (Ing. Švec – Penta, Praha) (C₆H₁₂, cHx)
- Deuterated chloroform for NMR analysis – chloroform-d 99,8 atom % D (Sigma Aldrich, Praha) (CDCl₃)
- Deuterated methanol for NMR analysis – Methanol-d₄ 99,8 atom % D (Sigma Aldrich, Praha) (CD₃OD)
- Diethyl ether f.a. (Ing. Švec – Penta, Praha) (Et₂O)
- Dioxane f.a. (Lach-Ner, Neratovice) (C₄H₈O₂)
- Ethanol 95% (Lihovar Chrudim, Chrudim) (EtOH)
- Ethyl acetate p. (Ing. Švec – Penta, Praha) (EtOAc)
- n-Hexane (Carlo Erba Reagents, Val-de-Reuil, France) (C₆H₁₄)
- Methanol f.a. (Ing. Švec – Penta, Praha) (MeOH)
• Petrol for medical purpose RN agreed with ČL and ČSN 656544 (Ing. Švec – Penta, Praha)
• Toluene f.a. (Ing. Švec – Penta, Praha) (C₆H₅CH₃, To)
• Water ultrapure

5.1.3 **Adsorbents for column chromatography, analytical and preparative thin layer chromatography**

• Aluminium oxide (Al₂O₃) neutral, for chromatography, 63 – 200 µm; Across (Lach-Ner, Neratovice). The activation of Al₂O₃ was performed at 200°C for 8 hours. After cooling, the active Al₂O₃ was deactivated by the addition of water (6 %) and mixed in a closed bowl for 1 hour.
• The silica gel: Silica gel 60 GF₂₅₄ (Merck Millipore, Praha) was used for the preparation of the molten layer on the sheets.
• Commercial analytical TLC sheets Silica gel GF₂₅₄ for TLC (Merck Millipore, Praha) – the size of the sheets was 20 × 20 cm. Plates with a 10 cm height were used for checking purposes (their width depended on the number of samples).
• Compounds on the plate were observed under UV light (254 and 366 nm) and visualized by spraying with Dragendorff’s reagent.

5.1.4 **Mobile phases used for the preparative and analytical TLC**

During the isolation, 10 different mobile phases were used for the preparative and analytical TLC.

MP-1: cHₓ:To:DEA (48:48:4)
MP-2: cHₓ:DEA (95:5)
MP-3: cHₓ:DEA (9:1)
MP-4: cHₓ:acetone:NH₄OH (30:60:2)
MP-5: cHₓ:EtOAc:DEA (90:5:5)
MP-6: cHₓ:To:EtOH:DEA (50:40:5:5)
MP-7: cHₓ:To:DEA (45:45:10)
MP-8: cHₓ:To:EtOH:DEA (40:40:10:10)
MP-9: To:EtOAc:DEA (55:40:5)
MP-10: cHₓ:EtOAc:DEA (80:10:10)
5.1.5  **Reagents used for the detection of the alkaloids**

- *Dragendorff's reagent* (according to Munier) was prepared as a stock solution by mixing solution A (1.7 g basic bismuth nitrate, 20 g tartaric acid dissolved in 80 ml of water) with solution B (32 g of potassium iodide in 80 ml of water) in the ratio 1:1. The solution for detection was prepared by blending 10 g of tartaric acid in 50 ml of water with 5 ml of the stock solution. Both the solution for detection and the stock solution were stored in the refrigerator at 4°C.

- *Mayer's reagent* was prepared by dissolving 5 g of potassium iodide in 30 ml of water. 1.35 g of mercuric chloride in powder was added to this solution. A precipitate occurred which was blended until dissolved. The final solution appeared to be either colourless or brightly yellow. The reagent was stored in the refrigerator at 4°C.

5.1.6  **Additional materials**

- Diatomite Celite C 535 John's Manville (Sigma Aldrich, Praha)
- Silica gel drying pearls (Ing. Švec – Penta, Praha)
- Sodium sulfate anhydrous f.a. (Ing. Švec – Penta, Praha)

5.1.7  **Instruments used for the isolation of alkaloids**

- Ultrasonic bath Sonorex Super 10P (Bandelin, Berlin, Germany)
- Vacuum evaporator for the half-operational use Laborota 20 Heidolph (Heidolph, Schwabach, Germany)
- Vacuum evaporator Buchi Rotavapor R-114 (Buchi Labortechnik AG, Switzerland)

5.2  **Materials and instruments used for the determination of biological activities**

5.2.1  **Chemicals used for the determination of the biological activities**

- Acetylthiocholine iodide f.a. (Sigma Aldrich, Praha) (ATChI)
- Berberine hydrochloride (Sigma Aldrich, Praha) ($C_{20}H_{18}NO_4Cl$)
- Butyrylthiocholine iodide f.a. (Sigma Aldrich, Praha) (BuTChI)
- Diethyl ether f.a. (Sigma Aldrich, Praha) (Et$_2$O)
• Monopotassium phosphate anhydrous f.a. (Lach-Ner, Neratovice) (KH₂PO₄)
• Sodium dihydrogen phosphate f.a. (Ing. Švec – Penta, Praha) (NaH₂PO₄·2H₂O)
• Dimethyl sulfoxide f.a. (Sigma Aldrich, Germany) (DMSO)
• 5,5’-dithiobis-(2-nitrobenzoic acid) ≥ 98% (Ellman's reagent) (Sigma Aldrich, Praha) (DTNB)
• Ethyl acetate f.a. (Sigma Aldrich, Germany) (EtOAc)
• Galanthamine hydrobromide > 98% (Changsha Organic Herb Inc., People Republic of China) (C₁₇H₂₁NO₃)
• Disodium phosphate anhydrous f.a. (Ing.Švec – Penta, Praha) (Na₂HPO₄)
• Huperzine A 98% (Tai’an zhonghui Plant Biochemical Co., Ltd., People Republic of China)
• Potassium chloride f.a. (Ing.Švec – Penta, Praha) (KCl)
• Sodium chloride f.a. (Lachema, Brno) (NaCl)
• Z-Gly-p-nitroanilide ≥ 99% (Sigma Aldrich, Praha) (C₁₃H₁₆N₄O₄)
• Z-Pro-prolinal ≥ 98% (Sigma Aldrich, Praha) (C₁₈H₂₂N₂O₄)

5.2.2 Preparation of buffer solutions
• 5mM Phosphate-buffered saline, pH 7.4 – prepared by mixing 57 ml of solution A with 283 ml of solution B and 300 ml of water. The stock solution A – 10 mM solution of NaH₂PO₄ (1.20 g of NaH₂PO₄ in 1 l of this solution). The stock solution B – 10 mM solution of Na₂HPO₄ (1.42 g of Na₂HPO₄ in 1 l of this solution).
• 5 mM Phosphate-buffered saline, pH 7.4, containing 150 mM solution of sodium chloride (8.766 g of sodium chloride f.a. is dissolved in 5 mM phosphate-buffered saline pH 7.4 and filled with this buffer solution to 1000 ml).
• 100 mM Phosphate-buffered saline, pH 7.4 – prepared by mixing 57 ml of solution A and 243 ml of solution B and 300 ml of water. The stock solution A is a 200 mM solution of NaH₂PO₄ (24.0 g of NaH₂PO₄ in 1 l of this solution). The stock solution B – 200 mM solution of Na₂HPO₄ (28.4 g of Na₂HPO₄ in 1 l of this solution).
Phosphate-buffered saline: 100 mM Sodium/Potassium phosphate-buffered saline, pH 7.4, containing 137 mM sodium chloride and 2.7 mM potassium chloride – solution A was prepared by mixing 0.68 g of KH$_2$PO$_4$ and 1.5 g sodium chloride and dissolved in 100 ml distilled water. Solution B was prepared by dissolving of 0.89 g of NaH$_2$PO$_4$ · 2 · H$_2$O and 1.5 g of NaCl in 100 ml of distilled water. 20 ml of solution A and 100 ml of solution B were blended.

The values of pH were tested in all of the buffered solutions. They were measured on the calibrated pH meter PHM 220.

5.2.3 Instruments used for the determination of biological activities

- Microplate ELISA reader EL800 (Bio-Tek Instruments, Inc., Winooski, Vermont, USA)
- Microplates with 96 sample wells (Fisher Scientific, Pardubice)
- Centrifuge AVANTI J-301 with rotor JA-30.50 (Beckman Coulter, Brea, California, USA)
- Centrifuge Boeco U-32R (Boeco, Hamburg, Germany) with rotor Hettich 1611 (Hettich, Tuttingen, Germany)
- pH meter PHM 220 (Radiometer, Copenhagen, Denmark)
- Polarimeter P3000 (A. Krüss Optronic, Hamburg, Germany)
- Reader Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, Vermont, USA)
- Statistical program GraphPad Prism 5.0 (GraphPad Software, San Diego, California, USA, 2006)

5.3 Plant material

The fresh bulbs of *Nerine bowdenii* Watson were obtained from the herbal dealer Lukon Glads (Sadská, Czech Republic). Botanical identification was performed by Prof. L. Opletal. A voucher specimen is deposited in the Herbarium of the Faculty of Pharmacy in Hradec Králové under number: CUFPH-16130/AL-254.
5.4 GC/MS analysis of alkaloidal extract of *Nerine bowdenii*

GC/MS analysis of the prepared alkaloid extracts was made within the scope of the screening study of plants from the Amaryllidaceae family conducted in our laboratory\(^5\)\(^6\)\(^7\). We have chosen *Nerine bowdenii* Watson for detailed phytochemical study – isolation of alkaloids used for other biological studies.

Fig. 10 shows the chromatogram of the summary alkaloid extract from the bulbs of *Nerine bowdenii* Watson, along with some identified alkaloids.
Fig. 10. Chromatogram of the summary alkaloid extract of *Nerine bowdenii* Watson.

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5.5 Extraction and isolation of alkaloids

Fresh bulbs (10 kg) were minced and exhaustively extracted with ethanol (EtOH) (96%, v/v, 3×) by boiling for 30 min under reflux; the combined extract was filtered and evaporated to dryness under reduced pressure. The crude extract (425 g) was acidified to pH 1.5 with 2% hydrochloric acid (HCl; 2.5 L), filtered, the filtrate defatted with diethyl ether (Et₂O; 3× 3.5 L), alkalized to pH 10 with a 25% solution of ammonia and exhaustively extracted with ethyl acetate (3× 3.5 L). The organic layer was evaporated to give 51 g of fluid residue. The obtained extract, which was Dragendorff positive, was further fractionated by CC on Al₂O₃ (2500 g), eluting with light petrol gradually enriched with CHCl₃ (20:80 - 10:90), and then CHCl₃ enriched with EtOH (99:1 – 50:50). Fractions of 500 mL were collected and monitored by TLC, yielding 157 fractions, which were combined into 8 fractions, and analyzed by GC-MS (Table 6).

Detailed process of the column chromatography is summed in the Table 5. There is a TLC analysis of the fractions in the Fig. 11.

| Table 5. The conditions of the column chromatography of the alkaloid extract |
|---------------------------------|----------------|
| Weight of the fluid residue     | 51 g           |
| Adsorbent for the slurry        | 200 g          |
| Adsorbent                       | Al₂O₃; 2500 g  |
| Dead volume                     | 1800 ml        |
| Fractions                       | 500 ml         |
| Dividing layer in the column    | 112 cm         |
| Layer with the alkaloid fraction| 15 cm          |
Fig. 11. TLC analysis of the acquired fractions
Table 6. The result of the column chromatography

<table>
<thead>
<tr>
<th>Sign</th>
<th>Joined fractions</th>
<th>Composition of eluent</th>
<th>Weight (g)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Fr. 1-25</td>
<td>Petrol:CHCl$_3$ (80:20)</td>
<td>0</td>
<td>without alkaloids</td>
</tr>
<tr>
<td>I</td>
<td>Fr. 26-32</td>
<td>Petrol:CHCl$_3$ (70:30)</td>
<td>2.483</td>
<td>brown, viscid</td>
</tr>
<tr>
<td>II</td>
<td>Fr. 33-38</td>
<td>Petrol:CHCl$_3$ (65:35)</td>
<td>4.285</td>
<td>brown, viscid</td>
</tr>
<tr>
<td></td>
<td>Fr. 39-71</td>
<td>Petrol:CHCl$_3$ (65:35)</td>
<td>6.598</td>
<td>russet, viscid</td>
</tr>
<tr>
<td></td>
<td>Fr. 72-104</td>
<td>Petrol:CHCl$_3$ (50:50)</td>
<td>7.275</td>
<td>russet, viscid</td>
</tr>
<tr>
<td></td>
<td>Fr. 105-112</td>
<td>Petrol:CHCl$_3$ (25:75)</td>
<td>1.387</td>
<td>russet, viscid</td>
</tr>
<tr>
<td>VI</td>
<td>Fr. 113-136</td>
<td>Petrol:CHCl$_3$ (20:80)</td>
<td>4.524</td>
<td>russet, viscid</td>
</tr>
<tr>
<td>VII</td>
<td>Fr. 137-144</td>
<td>Petrol:CHCl$_3$ (10:90)</td>
<td>1.024</td>
<td>russet, viscid</td>
</tr>
<tr>
<td>VIII</td>
<td>Fr. 145-157</td>
<td>CHCl$_3$ (100) CHCl$_3$:EtOH (50:50)</td>
<td>1.086</td>
<td>russet, viscid</td>
</tr>
</tbody>
</table>

Based on the TLC and GC/MS analysis, fraction 0 (0 g) was not put through another analysis due to its zero presence of alkaloids.

**Fraction I** (2.483 g) was processed via preparative TLC on the poured plates with the use of the mobile phase MP-1. The plates were developed in this mobile phase twice. Two zones, signed as I-a and I-b, were separated. The zone I-b was eluted with CHCl$_3$:EtOH in the ratio 1:1 and 2.104 g of a compound named NB-1 was obtained. After the structural analysis, it was identified as the belladine-type alkaloid belladine. We were not able to obtain any substances from the zone I-a due to small quantities.

**Fraction II** (4.285 g) was chromatographed by preparative TLC on the Merck commercial plates using mobile phase MP-2 once. Four zones were separated, labeled as II-a to II-d. Sub-fraction II-b was further processed by preparative TLC on the poured plates using the mobile phase MP-1, developed 2.5 times. Another four zones
were marked as II-b/1 – II/b-4. We were able to gain a compound named NB-2 by recrystallization with a mixture of CHCl₃:EtOH (1:1) from the zone II-b/2. NB-2 (1.571 g) was further analysed and identified as the crinine-type alkaloid undulatine. Chromatography of the sub-fraction II-c was performed by preparative TLC on Merck commercial plates with the mobile phase MP-3, twice. It was divided into 4 zones II-c/1 – II-c/4. A compound named NB-3 (235 mg) was obtained as russet crystals from the zone II-c/3 via recrystallization from the mixture CHCl₃:EtOH (1:1). This compound was identified as the lycorine-type alkaloid acetylcaranine. Other subfractions were mixed and stored for further analysis.

Preparative TLC of fraction III (6.598 g) in the mobile phase MP-4 gave 4 subfractions III-a – III-d. For further isolation, sub-fraction III-c and III-d was used. Sub-fraction III-c was processed by preparative TLC on the poured plate using the mobile phase MP-5 once and MP-3 6×. It was divided into 5 zones, labelled III-c/1 – III-c/5, and compounds NB-4 (gained from sub-fraction III-c/3) (339 mg) and NB-5 (obtained from sub-fraction III-c/5) (24 mg) were obtained. They were identified as the lycorine-type alkaloid caranine and the crinine-type alkaloid 11-O-acetylambelline. Sub-fraction III-d was further chromatographed by preparative TLC on the poured plates using the mobile phase MP-5 3×. 3 zones, marked III-d/1 – III-d/3, were obtained. By elution of the zones III-d/1 and III-d/2 with the mixture CHCl₃:EtOH (1:1), compounds NB-6 (784 mg) and NB-7 (971 mg) were obtained. They were put through structural analysis and identified as the crinine-type alkaloids buphanidrine and buphanisine.

Fraction IV (7.275 g) was crystallized from EtOH yielding 3.16 g of NB-8, identified as ambelline. The mother liquor of fraction IV was further treated by preparative TLC on the poured plates with mobile phase MP-6 3×. The fraction was divided into 5 zones, labeled as IV-a – IV-e. The zone IV-d gave a compound NB-9 (108 mg), which was obtained by crystallization from EtOH and identified as the crinine-type alkaloid buphanamine via structural analysis.

Fraction V (1.387 g) was processed via preparative TLC in the mobile phase MP-7 3×. It led to separation into 3 sub-fractions V-a – e. Preparative TLC of sub-fraction V-a in the mobile phase MP-8 gave 6 zones, marked as V-a/1 – V-a/6. The poured plates were developed once. The zone V-a/2 yielded a compound NB-10 (12 mg) by the elution with the mixture CHCl₃:EtOH (1:1) and the zone V-a/3 gave
compound NB-11 (18 mg) using the same procedure. The compounds were put through structural analysis and identified as the belladine-type alkaloids 4′-O-demethylbelladine and 6-O-demethylbelladine. Sub-fraction V-b was processed via preparative TLC on the poured plates using mobile phase MP-7. The plates were developed 3 times, which led to separation of 8 zones, labelled V-b/1 – V-b/8. Sub-fractions V-b/4, V-b/6 and V-b/8 were separated and eluted with the mixture CHCl₃:EtOH (1:1). This led to 3 compounds marked as NB-12 (17 mg), NB-13 (4.28 mg) and NB-14 (12 mg). These compounds were identified by structural analysis as the tazzetine-type alkaloid tazzetine and the crinine-type alkaloids epoxyambelline and 6-hydroxyundulatine. The remaining sub-fractions were not processed for their high number of minor alkaloids. They were mixed and stored for later processing.

Fraction VI (4.524 g) was treated by preparative TLC on the Merck commercial plates in the mobile phase MP-4. The plates were developed twice and 3 zones were isolated, marked as VI-a – VI-c. Subsequently, the sub-fraction VI-b (647 mg) was chromatographed in the mobile phase MP-9 once. This sub-fraction was divided into 3 zones VI-b/1 – VI-b/3 and the zone VI-b/2 gave compound NB-15 (468 mg), which was identified as the crinine-type alkaloid 1-O-acetylbulbisine. Recrystallization of the sub-fraction VI-c (1.025 g) gave NB-16 (652 mg), which was subsequently identified as the haemanthamine-type alkaloid haemanthamine via GC/MS and NMR analysis. The remaining sub-fractions and zones were mixed and stored for further analysis due to their content of many minor compounds.

Fraction VII (1.024 g) was processed via preparative TLC chromatography on the Merck commercial plates using the mobile phase MP-4. The plates were developed 3 times and separated into 3 zones, labelled as VII/1 – VII/3. The first zone (VII/1) was purified using preparative TLC on the poured plates. The plates were developed in the mobile phases MP-8 once and MP-3 twice. After elution with CHCl₃:EtOH (1:1), compound NB-17 (39 mg) was obtained, which was subsequently identified as the lycorine-type alkaloid 1-O-acetyllycorine. The zone VII/2 was treated in the same way using preparative TLC on the poured plate. It was developed in the mobile phase MP-9 once and the main zone was eluted with CHCl₃:EtOH (1:1). NB-18 (65 mg) was obtained. Structural analysis identified the compound as the crinine-type alkaloid crinamidine. GC/MS analysis of the zone VII/3 showed that it contained only one
compound, labelled **NB-19** (398 mg). This compound was eluted with CHCl₃:EtOH (1:1). Subsequent analysis identified it as the haemanthamine-type alkaloid hamayne.

**Fraction VIII** (1.086 g) was treated by preparative TLC on Merck commercial plates developed in the mobile phases **MP-4** once and, subsequently, **MP-10** twice. Altogether 5 zones were visible under UV light and after treatment with Dragendorff reagent. The two main zones **VIII/2** and **VIII/3** were eluted with CHCl₃:EtOH (1:1) yielding two compounds – **NB-20** (768 mg) from zone **VIII/2** and **NB-21** (32 mg) from zone **VIII/3**. Both compounds were put through structural analysis and identified as the crinine-type alkaloids crinine and powelline.

### 5.6 Structural identification of isolated alkaloids

#### 5.6.1 Gas chromatography-mass spectrometry analysis

GC/MS analysis of the isolated alkaloids was performed using an Agilent 7890A GC 5975 gas chromatograph joined with a mass spectrometer operating in electron ionisation mode at 70 eV. The separation itself was carried out on a HP-5 column. The temperature program was 100°C – 180°C (15°C·min⁻¹), 180°C (1 min), 180°C – 300°C (5°C·min⁻¹), 300°C (15 min). Helium was used as a carrier gas with a flow rate of 0.8 ml/min. The injector temperature was 280°C. 1 µl of alkaloid solution was injected (concentration 1 mg·ml⁻¹) in split mode (split ratio 1:10). The individual alkaloids were identified based on the comparison of their spectra with those available in the commerce NIST library (National Institute of Standards and Technology Library, USA), with data published in the literature and with the spectra of the reference compounds isolated earlier in our laboratory.

#### 5.6.2 Mass spectrometry with electrospray ionisation

MS (ESI) were measured for the compounds not ionisable via electron ionisation. The spectra were obtained with the help of an LC/MS Thermo Finnigan LCQDuo spectrometer with the electrospray ionisation in the cation mode. The ion trap was the analyser. The alkaloidal solution in MeOH (1 mg·ml⁻¹) was put directly on the probe.
5.6.3  **High resolution mass spectrometry**

ESI-HRMS were obtained with a Waters Synapt G2-Si hybrid mass analyzer of a quadrupole-time-of-flight (Q-TOF) type, coupled to a Waters Acquity I-Class UHPLC system.

5.6.4  **NMR analysis**

NMR spectra were measured on a VNMR S500 spectrometer operating at 499.87 MHz for $^1$H nucleus and 125.70 for $^{13}$C nucleus in CDCl$_3$ solutions at 25°C. The probe OneNMR was used for the radiation and detection of the signal. It is a broadband two-channel gradient probe with regulation of temperature. The chemical shifts were measured as δ values in parts per million (ppm). The values of the chemical shifts for CDCl$_3$ are: δ = 7.26 ppm for $^1$H atoms and δ = 77.0 ppm for $^{13}$C atoms. The data required by measurements are introduced in the order: chemical shift (δ), integrated intensity of $^1$H NMR spectrum, multiplicity (s: singlet, d: doublet, dd: doublet of doublets, t: triplet, q: quartet, m: multiplet) and the interaction constant $J$ (Hz).

The two-dimensional (2D) NMR experiments (gCOSY, gHSQC, gHMBC, NOESY) were measured in the standard sequences given by the Varian Company. The parameters for the measurements were set by the amount of the sample, the purity and molecular weight. A brief description of the methods is introduced below.

**gCOSY (gradient correlation spectroscopy):** this is a two-dimensional NMR technique where a crosspeak appears between protons which have a direct proton-proton bond through three bonds, $^3$$J_{HH}$.

**gHSQC (gradient heteronuclear single quantum coherence):** a two-dimensional NMR technique where one axis presents $^1$H and the other $^{13}$C. These spectra present a correlation between H and C through one bond.

**gHMBC (gradient heteronuclear multiple bond coherence):** a two-dimensional NMR technique which helps specifying a correlation of C with H through three bonds (in some cases even 2 or 4 bonds)

**NOESY (nuclear overhauser effect spectroscopy):** a two-dimensional NMR technique where crosspeaks appear between protons which are remote one from another to 6 Å. The intensity of the crosspeak drops with more remote distance of the protons in the area.
5.6.5 Optical rotation

Optical rotation of the alkaloids was measured in CHCl₃ solution on a P3000 polarimeter. Specific rotation was calculated according to the formula:

\[
[\alpha]_D^t = \frac{100 \times \alpha}{c \times l}
\]

t – temperature of the measurements; \( D \) – line of natrium light \( \lambda = 589.3 \text{ nm} \); \( \alpha \) – measured rotation \( [\circ] \); \( c \) – concentration of the measured alkaloid \( [\text{g} \cdot 100 \text{ ml}^{-1}] \); \( l \) – length of the cell \( [\text{dm}] \).

5.7 Biological assays of isolated alkaloids

5.7.1 Preparation of enzymes – HuAChE and HuBuChE

Enzymes were prepared from freshly drawn blood (taken from healthy volunteers), to which 2 mL 3.4% sodium citrate (w/v) per 18 mL blood was added, according to Steck and Kant, with slight modification. Plasma (HuBuChE) was removed from the whole blood by centrifugation at 4000 rpm in a Boeco U-32R centrifuge fitted with a Hettich 1611 rotor. Red blood cells were transferred to a 50 mL tubes and washed 3 times with 5 mM phosphate buffer (pH 7.4) containing 150 mM sodium chloride (centrifugation under same conditions). The washed erythrocytes were stirred with 5 mM phosphate buffer (pH 7.4) for 10 mins to ensure lysis. The lysed cells were dispensed for subsequent measurement. Activity of the enzyme preparations was measured immediately after preparation and adjusted with 5 mM phosphate buffer (pH 7.4) to reach the activity of blank sample \( A = 0.08 - 0.15 \) for AChE and \( A = 0.15 - 0.20 \) for BuChE.

5.7.2 HuAChE and HuBuChE inhibition assay

HuAChE and HuBuChE activities were determined using a modified Ellman’s method with acetylthiocholine iodide (ATChI) and butyrylthiocholine iodide (BuTChI) as substrates, respectively. Briefly, 8.3 μL of either blood cell lysate or plasma dilutions (at least 6 different concentrations), 283 μL of 5 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and 8.3 μL of the sample dilution in dimethyl sulfoxide (DMSO) (40 mM, 10 mM, 4 mM, 1 mM, 0.4 mM and 0 mM) were added to the semi-micro cuvette. The reaction was initiated by addition of 33.3 μL 10 mM substrate (ATChI or BuTChI). The final proportion of DTNB and substrate was 1:1. The increase of absorbance (ΔA) at 436 nm for AChE and 412 nm for BuChE was measured for 1
min at 37°C using a spectrophotometer (SynergyTM HT Multi-Detection Microplate Reader). Each measurement was repeated 6 times for every concentration of enzyme preparation. The % inhibition was calculated according to the formula: \[ I = 100 - \left( 100 \times \frac{\Delta A_{Bi}}{\Delta A_{Sa}} \right) \], where \( \Delta A_{Bi} \) is the increase of absorbance of the blank sample and \( \Delta A_{Sa} \) is the increase of absorbance of the measured sample. Inhibition potency of the tested compounds was expressed as IC\(_{50}\) value (concentration of inhibitor, which causes 50% cholinesterase inhibition).

### 5.7.3 Inhibition mechanism

The procedure for determination of the inhibition mechanism was similar to that for determination of IC\(_{50}\), with a difference in that uninhibited and inhibited processes were observed using three different concentrations of ATChI and BuTChI (20 µM, 40 µM, 60 µM). The dependence of absorbance (412 nm) vs. time was measured and the reaction rate was calculated for all reactions (uninhibited and inhibited). Then, a Lineweaver-Burk plot was constructed and \( K_m \) and \( V_m \) values were calculated. Each measurement was performed in duplicate.

### 5.7.4 Prolyl oligopeptidase assay

Prolyl oligopeptidase (POP; EC 3.4.21.26) was dissolved in phosphate buffered saline (PBS; 0.01 M Na/K phosphate buffer, pH 7.4, containing 137 mM NaCl and 2.7 mM KCl); the specific activity of the enzyme was 0.2 U/mL. The assay was performed in standard polystyrene 96-well microplates with a flat and clear bottom. Stock solutions of tested compounds were prepared in DMSO (10 mM). Dilutions (10\(^{-3}\) - 10\(^{-7}\) M) were prepared from the stock solution with deionized H\(_2\)O; the control was performed with the same DMSO concentration. POP substrate, (Z)-Gly-Pro-\(p\)-nitroanilide, was dissolved in 50% 1,4-dioxane (5 mM). For each reaction, PBS (170 µL), tested compound (5 µL), and POP (5 µL) were incubated for 5 min at 37°C. Then, substrate (20 µL) was added and the microplate was incubated for 30 min at 37°C. The formation of \(p\)-nitroanilide, directly proportional to the POP activity, was measured spectrophotometrically at 405 nm using a microplate ELISA reader (Multi-mode microplate reader Synergy 2, BioTek Instruments Inc., Vermont, USA). Inhibition
potency of tested compounds was expressed as IC$_{50}$ value (concentration of inhibitor which causes 50% POP inhibition).

5.7.5 In vitro cytotoxicity study

Cell culture and culture conditions

Selected human tumor and non-tumor cell lines {Jurkat (acute T cell leukemia), MOLT-4 (acute lymphoblastic leukemia), A549 (lung carcinoma), HT-29 (colorectal adenocarcinoma), PANC-1 (pancreas epithelioid carcinoma), A2780 (ovarian carcinoma), HeLa (cervix adenocarcinoma), MCF-7 (breast adenocarcinoma), SAOS-2 (osteosarcoma) and MRC-5 (normal lung fibroblasts)} were purchased from either ATCC (Manassas, USA) or Sigma-Aldrich (St. Louis, USA) and cultured according to the provider’s culture method guidelines. All cell lines were maintained at 37 °C in a humidified 5% carbon dioxide and 95% air incubator. Cells in the maximum range of either 10 passages for primary cell line (MRC-5), or in the maximum range of 20 passages for cancer cell lines (Jurkat, MOLT-4, A549, HT-29, PANC-1, A2780, HeLa, MCF-7 and SAOS-2) and in an exponential growth phase were used for this study.

Cell treatment

All the alkaloids evaluated and doxorubicin, used as positive control, were dissolved in dimethyl sulfoxide – DMSO (Sigma-Aldrich, St. Louis, USA) to prepare stock solutions with a concentration 10 - 50 mM based on their solubility. Stock solutions were freshly prepared before use in the experiments. For the experiments, the stock solutions were diluted with the appropriate culture medium to create final concentrations (10 µM for a single-dose alkaloid cytotoxicity screen and 1 µM for doxorubicin, used as a reference compound) making sure that the concentration of DMSO was < 0.1 % to avoid toxic effects on the cells. Control cells were sham-treated with a DMSO vehicle only (0.1 %; control).

WST-1 cytotoxicity assay

The WST-1 (Roche, Mannheim, Germany) reagent was used to determine the cytostatic effect of the test compounds. WST-1 is designed for the spectrophotometric quantification of cell proliferation, growth, viability and chemosensitivity in cell populations using a 96-well-plate format (Sigma, St.Louis, MO, USA). The principle of WST-1 is based on photometric detection of the reduction of tetrazolium salt to a colored formazan product. The cells were seeded at a previously established optimal
density (30000 Jurkat, 25000 MOLT-4, 500 A549, 1500 HT-29, 2000 PANC-1, 5000 A2780, 500 HeLa, 1500 MCF-7, 2000 SAOS-2 and 2000 MRC-5 cells/well) in 100 µL of culture medium, and adherent cells were allowed to reattach overnight. Thereafter, the cells were treated with 100 µl of either corresponding alkaloids or doxorubicin stock solutions to obtain the desired concentrations and incubated in 5% CO₂ at 37 °C. WST-1 reagent diluted 4-fold with PBS (50 µL) was added 48 hours after treatment. Absorbance was measured after 3 hours incubation with WST-1 at 440 nm. The measurements were performed in a Tecan Infinite M200 spectrometer (Tecan Group, Männedorf, Switzerland). All experiments were performed at least three times with triplicate measurements at each drug concentration per experiment. The viability was quantified as described in Havelek et al. according to the following formula: (%)

\[
\text{viability} = \left(\frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}}\right) \times 100,
\]

where \(A\) is the absorbance of the employed WST-1 formazan measured at 440 nm\(^{65}\). The viability of the treated cells was normalized to the viability of cells treated with 0.1 % DMSO (Sigma-Aldrich, St.Louis, MO, USA) as a vehicle control.

**MTT cytotoxicity assay**

Cell viability was measured using the MTT cytotoxicity assay originally developed by Mosman\(^{191}\). The assay for 3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2H-tetrazolium bromide (MTT) involves the quantification of viability of cells by the ability of living cells’ mitochondrial succinic dehydrogenase to reduce the yellow dye MTT to a blue insoluble product formazan. Briefly Caco-2, HT-29 and FHs-74 Int cells were seeded in 96-well plates at a density of \(2.5 \times 10^3\) cells per well. After 24 h, the cells were treated with two-fold serial diluted alkaloids (0.01-100 µM/mL Caco-2, HT-29 and 3.12-100 µM/ml FHs-74 Int) for 72 h. At the end of incubation, the spent medium in each well was replaced by fresh DMEM medium containing MTT (Sigma-Aldrich, St. Louis, MO, USA) reagent (1 mg/ml) and plates were incubated for an additional 2 hours at 37 °C. Two hours later, the culture supernatants were aspirated and the formazan product was dissolved in 100 µl of DMSO (Sigma-Aldrich, St. Louis, MO, USA). The absorbance was then measured at 555 nm using a Tecan Infinite M200 spectrometer (Tecan Group, Männedorf, Switzerland) and the % mortality for concentrations of each alkaloid were plotted and used to determine the 50% inhibitory concentration (IC\(_{50}\) value). Statistical analysis was performed using MagellanTM
software (Tecan Group, Männedorf, Switzerland) and Microsoft Office Excel 2003 (Microsoft, Redmond, WA, USA), from the data of three different experiments. Vinorelbine ditartrate salt hydrate (Sigma-Aldrich, Prague, Czech-Republic) was used as positive control in experiments.

**Statistical analysis**

The descriptive statistics of the results were calculated and the charts made in either Microsoft Office Excel 2010 (Microsoft, Redmond, WA, USA) or GraphPad Prism 5 biostatistics (GraphPad Software, La Jolla, CA, USA). In this study, all of the values were expressed as arithmetic means with SD of triplicates (n = 3), unless otherwise noted. The significant differences between the groups were analyzed using the Student's t-test and a P value ≤ 0.05 was considered statistically significant.
6 RESULTS

6.1 The overview of the isolated alkaloids and their structural analysis

6.1.1 NB-1: Belladine

**Summary formula: C\textsubscript{19}H\textsubscript{25}NO\textsubscript{3}**

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with that in the literature, the compound was identified as the belladine-type alkaloid belladine (Fig. 12\textsuperscript{55}).

![Fig. 12. Belladine](image)

**Molecular weight: 315.41**

**MS analysis**

EIMS \(m/z\) (%): 315(2), 194(70), 152(90), 151(100), 135(10), 121(18), 107(18)

**NMR analysis**

\(^1\)H NMR (500 MHz, CDCl\textsubscript{3})

7.10 (2H, d, \(J = 8.7\) Hz, H-4), 6.85 (1H, bs, H-3), 6.82 (2H, d, \(J = 8.7\) Hz, H-5), 6.81-6.77 (2H, m, H-6’, H-7’), 3.87 (3H, s, C5’-OCH\textsubscript{3}), 3.86 (3H, s, C4’-OCH\textsubscript{3}), 3.78 (3H, s, C6-OCH\textsubscript{3}), 3.49 (2H, s, H-1’), 2.77 (2H, t, \(J = 7.1\) Hz, H2), 2.60 (2H, t, \(J = 7.1\) Hz, H-1’), 2.28 (3H, s, N-CH\textsubscript{3})

\(^{13}\)C NMR (125 MHz, CDCl\textsubscript{3})

157.9, 148.9, 148.2, 132.5, 131.6, 129.6, 121.2, 113.7, 112.0, 110.6, 61.8, 58.9, 55.9, 55.9, 55.2, 41.9, 32.6

**Optical rotation**

Not having any chiral centre, the optical rotation was not measured.
6.1.2 NB-2: Undulatine

Summary formula: $\text{C}_{18}\text{H}_{21}\text{NO}_5$

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the crinine-type alkaloid undulatine (Fig. 13).

![Undulatine](image)

**Molecular weight: 331.36**

**MS analysis**

EIMS $m/z$ (%): 331(100), 316(6), 302(9), 300(6), 286(20), 272(5), 260(18), 258(40)

**NMR analysis**

$^1\text{H NMR (500 MHz, CDCl}_3$)

6.62 (1H, s, H-10), 5.89 (1H, d, $J = 1.5$ Hz, overlapped, OCH$_2$O), 5.88 (1H, d, $J = 1.5$ Hz, overlapped, OCH$_2$O), 4.27 (1H, d, $J = 17.6$ Hz, H-6), 4.00-3.97 (1H, m, H-3), 3.98 (3H, s, C7-OCH$_3$), 3.79 (1H, d, $J = 17.6$ Hz, H-6), 3.75 (1H, d, $J = 3.4$ Hz, H-1), 3.43 (3H, s, C3-OCH$_3$), 3.33 (1H, dd, $J = 3.4$, 2.4 Hz, H-2), 3.28 (1H, ddd, $J = 12.8$, 10.9, 4.7 Hz, H-12), 3.14 (1H, dd, $J = 13.6$, 3.3 Hz, H-4a), 2.85 (1H, ddd, $J = 12.8$, 9.1, 5.8 Hz, H-12), 2.43 (1H, ddd, $J = 12.5$, 10.9, 5.8 Hz, H-11), 2.04 (1H, ddd, $J = 12.5$, 9.1, 4.7 Hz, H-11), 1.91 (1H, brd, $J = 13.8$ Hz, H-4), 1.42 (1H, ddd, $J = 13.8$, 13.6, 3.0 Hz, H-4)
\(^{13}\)C NMR (125 MHz, CDCl\(_3\))

148.4, 141.0, 138.2, 133.5, 167.9, 100.7, 96.4, 74.6, 61.4, 59.1, 58.3, 57.7, 55.0, 53.5, 52.4, 41.7, 38.7, 24.7

**Optical rotation**

\([\alpha]^{21}_D = -32^\circ\) (c=0.5, CHCl\(_3\))

### 6.1.3 NB-3: Acetylcaranine

**Summary formula: C\(_{18}\)H\(_{19}\)NO\(_4\)**

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with that in the literature, the compound was identified as the lycorine-type alkaloid acetylcaranine (Fig. 14\(^{192,193}\)).

**Fig. 14. Acetylcaranine**

**Molecular weight: 313.35**

**MS analysis**

EIMS \(m/z\) (%): 313(80), 270(5), 253(20), 252(95), 250(15), 240(10), 225(40), 226(100)

**NMR analysis**

\(^1\)H NMR (500 MHz, CDCl\(_3\))

6.75 (1H, s, H-11), 6.73 (1H, s, H-8), 5.95-5.94 (1H, m, H-1), 5.94 (1H, d, \(J = 1.2\) Hz, overlapped, OCH\(_2\)O), 5.93 (1H, d, \(J = 1.2\) Hz, overlapped, OCH\(_2\)O), 5.59 (1H, d, \(J = 2.3\) Hz, H-3), 4.20 (1H, d, \(J = 14.0\) Hz, H-7), 3.99 (1H, d, \(J = 14.0\) Hz, H-7), 3.43 (1H, ddd, \(J = 10.2, 7.3, 4.9\) Hz, H-5), 3.39 (1H, d, \(J = 11.3\))
Hz, H-13), 3.06-3.00 (1H, m, H-5), 2.89 (1H, d, J = 11.3 Hz, H-12), 2.78-2.71 (2H, m, H-11), 2.70 (1H, bd, J = 19.9 Hz, H-2), 2.41 (1H, bd, J = 19.9 Hz, H-2), 1.92 (3H, s, OCOCH<sub>3</sub>)

<sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>)

172.1, 149.0, 148.3, 136.9, 129.0, 127.9, 118.3, 108.5, 105.9, 102.7, 67.3, 62.7, 56.0, 54.9, 42.7, 33.9, 29.6, 20.9

Optical rotation

\([\alpha]^{22.5}_D = -177.5^\circ \text{ (c=0.8, CHCl}_3\text{)}\)

6.1.4 NB-4: Caranine

Summary formula: C<sub>16</sub>H<sub>17</sub>NO<sub>3</sub>

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with that in the literature, the compound was identified as the lycorine-type alkaloid caranine (Fig. 15)<sup>91</sup>.

Fig. 15. Caranine

Molecular weight: 271.31

MS analysis

EIMS m/z (%): 271(86), 270(46), 252(52), 250(10), 240(12), 227(48), 226(100), 215(5)

NMR analysis

<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)

6.82 (1H, s, H-11), 6.58 (1H, s, H-8), 5.90 (1H, d, J = 16.1 Hz, overlapped, OCH<sub>2</sub>O), 5.90 (d, J = 16.1 Hz, OCH<sub>2</sub>O), 5.45-5.41 (1H, m, H-3), 4.76-4.70 (1H, m, H-1), 4.13 (1H, d, J = 14.0 Hz, H-7) 3.52 (1H, d, J = 14.0 Hz, H-7), 3.35-
3.31 (1H, m, H-5), 2.78 (1H, d, J = 10.7, 2.6 Hz, H-13), 2.61-2.59 (2H, m, H-2),
2.59-2.50 (2H, m, H-4), 2.41 (1H, dd, J = 10.7, 2.6 Hz, H-12), 2.35-2.31 (1H, m, H-5)

$^1^3^C$ NMR (125 MHz, CDCl$_3$)
146.6, 146.3, 139.0, 130.5, 127.8, 114.5, 107.8, 104.6, 101.0, 64.9, 60.9, 57.2,
53.9, 45.1, 35.3, 28.6

Optical rotatory
$[\alpha]^3^0_D = -203.1^\circ$ (c=0.9, CHCl$_3$)

6.1.5 NB-5: 11-O-Acetylambelline

Summary formula: C$_{20}$H$_{23}$NO$_6$

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the crinine-type alkaloid 11-O-acetylambelline (Fig. 16)\textsuperscript{55}.

Fig. 16. 11-O-Acetylambelline

Molecular weight: 373.40

MS analysis
EIMS m/z (%): 373(100), 358(15), 342(15), 330(10), 314(70), 313(69), 298(30),
282(55), 270(25)
NMR analysis

$^1$H NMR (500 MHz, CDCl$_3$)
6.51 (1H, d, $J = 10.0$ Hz, H-1), 6.43 (1H, s, H-10), 6.02 (1H, ddd, $J = 10.0$, 5.5, 1.0 Hz, H-2), 5.85 (2H, s, OCH$_2$O), 5.11 (1H, dd, $J = 8.0$, 4.0 Hz, H-11), 4.33 (1H, d, $J = 17.5$ Hz, H-6), 3.97 (3H, s, C7-OCH$_3$), 3.86 (1H, d, $J = 17.5$ Hz, H-6), 3.83 (1H, ddd, $J = 5.5$, 4.0, 2.0 Hz, H-3), 3.78 (1H, dd, $J = 14.0$, 8.0, H-12), 3.43 (1H, dd, $J = 13.5$, 3.0 Hz, H-4a), 3.32 (3H, s, C3-OCH$_3$), 2.72 (1H, ddd, $J = 14.0$, 4.0, 1.5 Hz, H-12), 2.16 (1H, brd, $J = 13.5$ Hz, H-4), 1.81 (3H, s, OCOCH$_3$), 1.70 (1H, ddd, $J = 13.5$, 13.5, 4.0 Hz, H-4)

$^{13}$C NMR (125 MHz, CDCl$_3$)
171.5, 148.2, 141.0, 134.3, 134.2, 132.3, 126.7, 117.9, 101.0, 99.7, 87.9, 72.7, 64.0, 60.0, 59.6, 59.3, 57.0, 48.0, 29.3, 21.4

Optical rotation

$\[
\alpha \]^{25}_{D} = +25^\circ$ (c=0.3, MeOH)

### 6.1.6 NB-6: Buphanidrine

**Summary formula: C$_{18}$H$_{21}$NO$_4$**

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the crinine-type alkaloid buphanidrine(Fig. 17)$^{194}$.

![Fig. 17. Buphanidrine](image-url)
Molecular weight: 315.36

MS analysis
EIMS m/z (%): 315(100), 300(26), 287(30), 272(10), 260(45), 245(60), 231(30), 228(22)

NMR analysis

$^1$H NMR (500 MHz, CDCl$_3$)
6.50 (1H, s, H-10), 6.42 (1H, d, $J = 10.0$ Hz, H-1), 5.95 (1H, dd, $J = 10.0$, 5.0 Hz, H-2), 5.81 (2H, s, OCH$_2$O), 4.35 (1H, d, $J = 17.0$ Hz, H-6), 3.95 (1H, d, $J = 17.0$ Hz, H-6), 3.92 (3H, s, C7-OCH$_3$), 3.79-3.74 (1H, m, H-3), 3.66-3.63 (1H, m, H-12), 3.50 (1H, dd, $J = 13.5$, 3.5 Hz, H-4a), 3.29 (3H, s, C3-OCH$_3$), 2.99 (1H, ddd, $J = 13.0$, 9.0, 6.0 Hz, H-12), 2.46 (1H, brd, $J = 13.5$ Hz, H-4), 2.20 (1H, ddd, $J = 12.5$, 9.0, 4.0 Hz, H-11), 1.99 (1H, ddd, $J = 12.5$, 10.5, 6.0 Hz, H-11), 1.60 (1H, ddd, $J = 13.5$, 13.5, 4.0 Hz, H-4)

$^{13}$C NMR (125 MHz, CDCl$_3$)
148.2, 140.8, 138.6, 133.4, 132.1, 125.5, 115.8, 100.6, 96.9, 72.2, 62.8, 59.1, 58.1, 56.5, 53.3, 44.3, 43.4, 28.0

Optical rotation

$[\alpha]_{25}^\text{D} = -10.1^\circ$ (c=0.3, CH$_3$OH)

6.1.7 NB-7: Buphanisine

Summary formula: C$_{17}$H$_{19}$NO$_3$

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the crinine-type alkaloid buphanisine (Fig. 18).
Molecular weight: 285.34

MS analysis

EIMS m/z (%): 285(100), 270(23), 253(25), 230(28), 215(80), 201(22), 187(20), 157(15)

NMR analysis

$^1$H NMR (500 MHz, CDCl$_3$)

6.86 (1H, s, H-10), 6.57 (1H, d, $J = 10.0$ Hz, H-1), 6.51 (1H, s, H-7), 6.03 (1H, dd, $J = 10.0$, 5.2 Hz, H-2), 5.92-5.90 (2H, m, OCH$_2$O), 4.52 (1H, d, $J = 16.8$ Hz, H-6), 3.90 (1H, d, $J = 16.8$ Hz, H-6), 3.86-3.83 (1H, m, H-3), 3.56 (1H, brd, $J = 13.0$ Hz, H-12), 3.50 (1H, dd, $J = 13.5$, 3.6 Hz, H-4a), 3.37 (3H, s, C3-OCH$_3$), 3.02 (1H, ddd, $J = 13.0$, 9.0, 6.0 Hz, H-12), 2.33 (1H, brd, $J = 13.5$ Hz, H-4), 2.23 (1H, ddd, $J = 12.3$, 9.0, 4.0 Hz, H-11), 2.01 (1H, ddd, $J = 12.3$, 10.8, 6.0 Hz, H-11), 1.64 (1H, ddd, $J = 13.5$, 12.5, 3.9 Hz, H-4)

$^{13}$C NMR (125 MHz, CDCl$_3$)

146.5, 146.1, 137.5, 131.9, 125.8, 124.3, 107.0, 103.1, 101.0, 72.2, 63.4, 61.6, 56.7, 53.2, 44.6, 43.4, 29.0

Optical rotation

$[\alpha]_{D}^{23} = -31^\circ$ (c=1, CHCl$_3$)
6.1.8 NB-8: Ambelline

Summary formula: C_{18}H_{21}NO_{5}

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the crinine-type alkaloid ambelline (Fig. 19)\textsuperscript{196,197}.

Molecular weight: 331.36

MS analysis

EIMS m/z (%): 331(100), 316(5), 299(30), 287(55), 270(25), 260(50), 257(35), 255(23), 241(27)

NMR analysis

\textsuperscript{1}H NMR (500 MHz, \(\delta\), CDCl\textsubscript{3}, 25 °C)

6.60 (1H, s, H-10), 6.51 (1H, d, \(J = 10.1\) Hz, H-1), 6.05 (1H, dd, \(J = 10.1, 5.3\) Hz, H-2), 5.91 (1H, d, \(J = 1.4\) Hz, overlapped, OCH\textsubscript{2}O), 5.90 (1H, d, \(J = 1.4\) Hz, overlapped, OCH\textsubscript{2}O), 4.40 (1H, bs, H-11), 4.36 (1H, d, \(J = 17.3\) Hz, H-6), 4.00 (3H, s, C7-OCH\textsubscript{3}), 3.93 (1H, d, \(J = 17.3\) Hz, H-6), 3.87-3.80 (2H, m, H3, H-12), 3.50 (1H, dd, \(J = 13.6, 3.9\) Hz, H-4a), 3.35 (3H, s, C3-OCH\textsubscript{3}), 2.56 (1H, dd, \(J = 13.6, 3.9\) Hz, H-12), 2.29 (1H, brd, \(J = 12.2\) Hz, H-4), 1.69 (1H, td, \(J = 13.7, 4.0\) Hz, H-4)

\textsuperscript{13}C NMR (125 MHz, \(\delta\), CDCl\textsubscript{3}, 25 °C)

148.6, 141.0, 134.5, 131.3, 130.8, 126.5, 116.0, 100.9, 100.3, 85.1, 71.9, 63.1, 61.8, 59.2, 58.7, 56.7, 48.2, 28.0
Optical rotation
\[ \alpha^{25}_D = +81^\circ \; (c=0.56, \text{CHCl}_3) \]

6.1.9 NB-9: Buphanamine

Summary formula: C_{17}H_{19}NO_4

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the crinine-type alkaloid buphanamine (Fig. 20)\textsuperscript{192,198,199}.

![Fig. 20. Buphanamine](image)

**Molecular weight: 301.34**

**MS analysis**

EIMS m/z (%): 301(100), 286(12), 284(14), 272(14), 256(18), 231(25), 218(18), 204(18)

**NMR analysis**

\(^1\text{H NMR (500 MHz, CDCl}_3\))

6.58 (1H, s, H-10), 6.01-5.96 (1H, m, H-2), 5.88 (1H, d, \( J = 17.3 \) Hz, overlapped, OCH\textsubscript{2}O), 5.87 (1H, d, \( J = 17.3 \) Hz, overlapped, OCH\textsubscript{2}O), 5.84 (1H, ddd, \( J = 10.5, 4.4, 2.9 \) Hz, H-3), 4.72 (1H, d, \( J = 5.4 \) Hz, H-1), 4.16 (1H, d, \( J = 17.2 \) Hz, H-6), 3.97 (3H, s, C7-OCH\textsubscript{3}), 3.81 (1H, d, \( J = 17.2 \) Hz, H-6), 3.44 (1H, t, \( J = 8.2 \) Hz, H-4a), 3.39 (1H, ddd, \( J = 12.6, 10.2, 2.6 \) Hz, H-12), 2.75 (1H, ddd, \( J = 12.6, 8.3, 7.6 \) Hz, H-12), 2.58 (1H, dddd, \( J = 19.7, 8.0, 4.5, 1.7 \) Hz, H-4), 2.00-1.91 (2H, m, H-4, H-11), 1.87-1.81 (1H, m, H-11)
C NMR (125 MHz, CDCl₃)
148.6, 140.7, 137.0, 133.6, 128.6, 125.6, 117.6, 100.7, 98.2, 64.4, 59.3, 59.1, 56.9, 51.5, 48.3, 38.6, 28.2

Optical rotation
\([\alpha]_{D}^{22} = -169° \ (c=0.0009, \text{CHCl}_3)\)

6.1.10 NB-10: 4'-O-Demethylbelladine

Summary formula: C₁₈H₂₃NO₃

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the belladine-type alkaloid 4'-O-demethylbelladine(Fig. 21)²⁰⁰. The important correlations obtained from the gHMBC experiment are shown in Fig. 22.

Molecular weight: 301.38

MS analysis
EIMS m/z (%): 301(1), 181(70), 137(100), 121(10), 107(15), 93(15)
NMR analysis

$^1$H NMR (500 MHz, CDCl$_3$)

7.13–7.09 (1H, m, H-4), 7.13–7.09 (1H, m, H-8), 7.00–6.97 (1H, m, H-3’), 6.92–6.88 (1H, m, H-7’), 6.86–6.81 (1H, m, H-5), 6.86–6.81 (1H, m, H-7), 6.86–6.81 (1H, m, H-6’), 3.90 (3H, s, C5’-OCH$_3$), 3.80–3.77 (2H, m, H-1’), 3.79 (3H, s, C6-OCH$_3$), 2.98–2.91 (2H, m, H-2), 2.89–2.82 (2H, m, H-1), 2.48 (3H, s, N-CH$_3$)

$^{13}$C NMR (125 MHz, CDCl$_3$)

158.3, 146.9, 145.8, 130.4, 129.7, 129.7, 126.7, 121.9, 116.3, 114.1, 114.0, 110.8, 60.5, 57.8, 56.0, 55.3, 40.6, 31.3

Optical rotation

As there was not a chiral centre, the optical rotation was not measured.

6.1.11 NB-11: 6-O-Demethylbelladine

Summary formula: C$_{18}$H$_{23}$NO$_3$

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the belladine-type alkaloid 6-O-demethylbelladine (Fig. 23$^{200}$). The important correlations obtained from the gHMBC experiment are shown in Fig. 24.

![Fig. 23. 6-O-Demethylbelladine](image)

![Fig. 24. Key gHMBC correlation of 6-O-demethylbelladine](image)
Molecular weight: 301.38

MS analysis
EIMS m/z (%): 301(1), 194(68), 151(100), 135(12), 121(15), 107(17)

NMR analysis

$^1$H NMR (500 MHz, CDCl$_3$)
7.00-6.94 (1H, m, H-4), 7.00-6.94 (1H, m, H-8), 7.00-6.94 (1H, m, H-3’), 6.86-6.79 (1H, m, H-6’), 6.86-6.79 (1H, m, H-7’), 6.76-6.72 (1H, m, H-5), 6.76-6.72 (1H, m, H-7), 3.88 (3H, s, C5’-OCH$_3$), 3.85 (3H, s, C4’-OCH$_3$), 3.72-3.76 (2H, m, H-1’), 2.86-2.73 (2H, m, H-1), 2.86-2.73 (2H, m, H-2), 2.40 (3H, s, N-CH$_3$)

$^{13}$C NMR (125 MHz, CDCl$_3$)
155.0, 149.1, 148.7, 130.4, 129.7, 129.7, 128.4, 122.0, 115.5, 115.5, 112.6, 110.8, 61.1, 58.3, 55.9, 55.9, 41.0, 31.6

Optical rotation
By virtue of not having any chiral centre, the optical rotation was not measured.

6.1.12 NB-12: Tazettine

Summary formula: C$_{18}$H$_{21}$NO$_5$

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the tazettine-type alkaloid tazettine(Fig. 25)

Fig. 25. Tazettine
Molecular weight: 331.36

MS analysis

EIMS $m/z$ (%): 331(20), 316(20), 298(25), 247(100), 230(10)

NMR analysis

$^1$H NMR (500 MHz, CDCl$_3$)

6.86 (1H, s, H-12), 6.50 (1H, s, H-9), 6.14 (1H, dt, $J = 10.4$, 1.4 Hz, H-2), 5.90 (2H,s, OCH$_2$O), 5.61 (1H, dt, $J = 10.4$, 1.8 Hz, H-1), 4.96 (1H, dd, $J = 14.7$, 0.5 Hz, H-8), 4.63 (1H, d, $J = 14.7$ Hz, H-8), 4.11-4.17 (1H, m, H-3), 3.46 (3H, s, OCH$_3$), 3.30 (1H, d, $J = 10.6$ Hz, H-6), 2.85-2.89 (1H, m, H-6a), 2.68 (1H, d, $J = 10.6$ Hz, H-6), 2.40 (3H, s, N-CH$_3$), 2.19-2.27 (1H, m, H-4), 1.63 (1H, ddd, $J = 13.7$, 10.1, 2.4 Hz, H-4)

$^{13}$C NMR (125 MHz, CDCl$_3$)

146.6, 146.4, 130.6, 128.7, 128.0, 125.5, 109.3, 104.0, 102.0, 100.9, 72.9, 70.0, 65.5, 62.0, 56.1, 49.9, 42.0, 26.7

Optical rotation

$[\alpha]^{25}_D = +67.5^\circ$ (c=0.86, CHCl$_3$)

6.1.13 NB-13: 1,2β-Epoxyambelline

Summary formula: C$_{18}$H$_{21}$NO$_6$

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the crinine-type alkaloid 1,2β-epoxyambelline (Fig. 26).
Fig. 26. 1,2β-Epoxyambelline

**Molecular weight:** 347.36

**MS analysis**

EIMS $m/z$ (%): 347(33), 318(100), 274(25), 244(24), 231(27), 205(47), 189(14), 173(11), 115(18).

**NMR analysis**

$^1$H NMR (500 MHz, CDCl$_3$)

6.71 (1H, s, H-10), 5.97-5.95 (2H, m, OCH$_2$O), 5.03 (1H, bs, H-11), 4.55 (1H, d, $J = 16.3$ Hz, H-6), 4.11 (1H, d, $J = 16.3$ Hz, H-6), 4.04 (3H, s, C7-OCH$_3$), 4.02-4.00 (2H, m, H3, H-12), 3.69 (1H, d, $J = 3.7$ Hz, H-1), 3.57-3.49 (1H, m, H-4a), 3.43 (3H, s, C3-OCH$_3$), 3.37-3.34 (1H, m, H-2), 2.78-2.70 (1H, m, H-12), 2.58-2.41 (1H, m, H-4), 1.55 (1H, ddd, $J = 13.8, 11.5, 2.4$ Hz, H-4)

$^{13}$C NMR (125 MHz, CDCl$_3$)

149.4, 141.0, 134.7, 129.8, 113.0, 101.4, 100.0, 77.2, 73.5, 62.1, 59.4, 58.4, 58.1, 57.7, 54.1, 51.8, 46.1, 22.9

**Optical rotation**

$[\alpha]_{D}^{20} = -14.6^\circ$ (c=1.15, CH$_3$OH)
6.1.14 **NB-14: 6α-Hydroxyundulatine**

**Summary formula:** $\text{C}_{18}\text{H}_{21}\text{NO}_6$

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the crinine-type alkaloid 6α-hydroxyundulatine (Fig. 27).\(^{194}\)

![Fig. 27. 6α-Hydroxyundulatine](image)

**Molecular weight:** 347.36

**MS analysis**

Elms $m/z$ (%): 347(47), 318(3), 276(44), 274(22), 256(39), 246(21), 231(27), 219(96), 204(38), 189(18), 173(14), 159(9), 115(27), 56(100).

**NMR analysis**

$^1$H NMR (500 MHz, CDCl$_3$)

6.61 (1H, s, H-10), 5.90-5.88 (2H, d, $J = 1.5$ Hz, OCH$_2$O), 5.21 (1H, s, H-6), 4.03 (3H, s, C7-OCH$_3$), 3.96 (1H, dd, $J = 3.0$, 2.5 Hz, H-3), 3.73 (1H, d, $J = 3.4$ Hz, H-1), 3.65 (dd, $J = 13.5$, 3.5 Hz, H-4a), 3.39 (3H, s, C3-OCH$_3$), 3.32 (1H, ddd, $J = 3.4$, 2.5, 1.0 Hz, H-2), 3.17 (1H, ddd, $J = 13.5$, 10.5, 4.5 Hz, H-12), 2.75 (1H, ddd, $J = 13.5$, 9.5, 6.0 Hz, H-12), 2.39 (1H, ddd, $J = 12.0$, 10.5, 6.0 Hz, H-11), 1.87-1.83 (1H, m, H-4), 1.78 (1H, ddd, $J = 12.0$, 9.5, 4.5 Hz, H-11), 1.35 (1H, ddd, $J = 14.0$, 13.5, 3.0 Hz, H-4)

$^{13}$C NMR (125 MHz, CDCl$_3$)

149.2, 142.8, 138.8, 134.4, 119.2, 100.8, 96.6, 85.2, 74.4, 59.7, 57.2, 55.0, 54.6, 53.8, 46.1, 41.7, 35.5, 24.3
Optical rotation

\[ \alpha^{20}_D = +8.4^\circ \ (c=0.53, \text{MeOH}) \]

6.1.15 NB-15: 1-O-Acetylbulbisine

Summary formula: C_{19}H_{23}NO_6

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the crinine-type alkaloid 1-O-acetylbulbisine (Fig. 28):

\[ \text{Fig. 28. 1-O-Acetylbulbisine} \]

Molecular weight: 361.39

MS analysis

EIMS m/z (%): 361(100), 314(24), 302(64), 272(36), 254(31), 231(23), 202(21), 191(12), 115(13), 43(56)

NMR analysis

\( ^1H \text{ NMR (500 MHz, CDCl}_3 \)

6.13 (1H, s, H-10), 5.84 (1H, d, \( J = 18.1 \text{ Hz, overlapped, CH}_2\text{OCH}_3 \)), 5.84 (1H, d, \( J = 18.1 \text{ Hz, overlapped, CH}_2\text{OCH}_3 \)), 5.62 (1H, d, \( J = 2.9 \text{ Hz, H-1} \)), 4.13 (1H, d, \( J = 17.1 \text{ Hz, H-6} \)), 4.05 – 4.01 (1H, m, H-2), 3.98 (3H, s, C7-OCH_3), 3.75 (1H, d, \( J = 17.1 \text{ Hz, H-6} \)), 3.35-3.27 (1H, m, H-12), 3.17 (1H, dd, \( J = 11.2, 5.4 \text{ Hz, H-4a} \)), 3.04-2.96 (1H, m, H-11), 2.75-2.67 (1H, m, H-12), 1.93 (3H, s, OCOCH_3), 1.86-1.79 (1H, m, H-11), 1.79 – 1.61 (2H, m, H-4)
\[^{13}\text{C NMR (125 MHz, CDCl}_3\text{)}\]

170.5, 148.2, 140.4, 139.2, 133.0, 117.5, 100.4, 96.9, 71.5, 68.0, 64.1, 59.1, 57.7, 51.2, 46.5, 37.9, 26.8, 21.2, 21.1

Optical rotation

\[\left[\alpha\right]_D^{\text{23}} = +32^\circ \text{ (c=0.5, CH}_3\text{OH)}\]

**6.1.16 NB-16: Haemanthamine**

**Summary formula: C\(_{17}\)H\(_{19}\)NO\(_4\)**

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the haemanthamine-type alkaloid haemanthamine (Fig. 29)\(^{196}\).

![Fig. 29. Haemanthamine](image)

**Molecular weight: 301.34**

**MS analysis**

EIMS \(m/z\) (%): 301(M\(^+\), 15), 272(100), 240(18), 257(20), 211(16), 181(23)

**NMR analysis**

\[^1\text{H NMR (500 MHz, CDCl}_3\text{)}\]

6.74 (1H, s, H-10), 6.41 (1H, s, H-7), 6.36 (1H, d, \(J = 10.2\) Hz, H-1), 6.25 (1H, dd, \(J = 10.2, 4.7\) Hz, H-2), 5.81 (1H, s, OCH\(_2\)O), 5.76 (1H, s, OCH\(_2\)O), 4.25 (1H, d, \(J = 17.1\) Hz, H-6), 3.96 (1H, dd, \(J = 6.7, 3.3\) Hz, H-11), 3.82 (1H, m, H-3), 3.72 (1H, d, \(J = 17.1\) Hz, H-6), 3.36 (3H, s, C3-OCH\(_3\)), 3.25-3.30 (2H, m, H-12, H-4a), 3.19 (1H, dd, \(J = 14.1, 3.3\) Hz, H-12), 2.11 (1H, ddd, \(J = 13.8, 5.4, 1.8\) Hz, H-4), 1.96 (1H, m, H-4)
\[^{13}\text{C}\] NMR (125 MHz, CDCl\(_3\))

146.5, 146.3, 136.1, 135.4, 126.7, 123.6, 106.9, 103.2, 100.9, 80.1, 76.1, 66.2, 63.5, 61.3, 55.8, 50.3, 30.3

Optical rotation

\[^{25}\alpha\]^D = +37° (c=0.29 CHCl\(_3\))

6.1.17 NB-17: 1-O-Acetyllycorine

Summary formula: C\(_{18}\)H\(_{19}\)NO\(_5\)

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the lycorine-type alkaloid 1-O-acetyllycorine (Fig. 30).\(^{201}\)

![Fig. 30. 1-O-Acetyllycorine](image)

Molecular weight: 329.35

MS analysis

EIMS \(m/z\) (100%): 329(52), 268(37), 250(24), 240(9), 226(100), 192(3)

NMR analysis

\(^1\text{H}\) NMR (500 MHz, CDCl\(_3\))

6.64 (1H, s, H-10), 6.56 (1H, s, H-7), 5.91 (2H, s, OCH\(_2\)O), 5.59 (1H, s, H-1), 5.55-5.52 (1H, m, H-3), 4.18-4.16 (1H, m, H-2), 4.14 (1H, d, \(J = 14.0\) Hz, H-6), 3.52 (1H, d, \(J = 14.0\) Hz, H-6), 3.35 (1H, ddd, \(J = 9.3, 4.9, 4.5\) Hz, H-12), 2.86 (1H, d, \(J = 10.4\) Hz, H-10b), 2.77 (1H, d, \(J = 10.4\) Hz, H-4a), 2.65-2.60 (2H, m, H-11), 2.40 (1H, ddd, \(J = 9.3, 8.3, 7.9\) Hz, H-12), 1.93 (3H, s, OCOCH\(_3\))
$^{13}$C NMR (125 MHz, CDCl$_3$)

170.8, 146.5, 146.2, 143.8, 129.2, 127.0, 117.3, 107.3, 104.8, 100.9, 72.6, 69.5, 61.5, 56.7, 53.7, 39.2, 28.6, 21.0

Optical rotation

$[\alpha]_{D}^{20} = -118.9^\circ$ (c=0.09, CH$_3$OH)

6.1.18 NB-18: Crinamide

Summary formula: C$_{17}$H$_{19}$NO$_5$

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the crinine-type alkaloid crinamidine (Fig. 31)$^{154,194,202}$.

Fig. 31. Crinamide

Molecular weight: 317.34

MS analysis

EIMS m/z (%): 317(25), 288(100), 274(5), 259(22), 258(25), 243(28), 244(30), 230(25), 217(40), 205(40)

NMR analysis

$^1$H NMR (500 MHz, CDCl$_3$)

6.61 (1H, s, H-10), 5.86-5.84 (2H, m, OCH$_2$O), 4.48 (1H, ddd, $J = 3.0, 2.5, 2.0$ Hz, H-3), 4.19 (1H, d, $J = 17.5$ Hz, H-6), 3.95 (3H, s, C7-OCH$_3$), 3.75 (1H, d, $J = 4.0$ Hz, H-1), 3.71 (1H, d, $J = 17.5$ Hz, H-6), 3.26 (1H, ddd, $J = 4.0, 2.5, 1.5$ Hz, H-2), 3.17 (1H, dd, $J = 12.5, 5.5$ Hz, H-4a), 3.17 (1H, ddd, $J = 12.5, 10.5$, 10.5,
5.0 Hz, H-12), 2.77 (1H, ddd, J = 12.5, 9.0, 5.5 Hz, H-12), 2.37 (1H, ddd, J = 12.5, 10.5, 5.5 Hz, H-11), 2.0 (1H, ddd, J = 12.5, 9.0, 5.0 Hz, H-11), 1.61 (1H, ddd, J = 13.5, 5.5, 2.0, 1.5 Hz, H-4), 1.56 (1H, ddd, J = 13.5, 12.5, 3.0 Hz, H-4) 
\[^{13}\text{C NMR (125 MHz, CDCl}_3\)\]
148.1, 141.1, 138.7, 133.4, 117.6, 100.7, 96.4, 65.5, 61.0, 59.1, 58.6, 56.4, 53.8, 52.5, 41.6, 39.2, 29.7

**Optical rotation**

\[\left[\alpha\right]_{D}^{21} = -5^o \text{ (c= 0.2, CHCl}_3\)\]

6.1.19 NB-19: Hamayne

**Summary formula: C\(_{16}\)H\(_{17}\)NO\(_4\)**

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as haemanthamine-type alkaloid hamayne (Fig. 32).\(^{196}\)

![Fig. 32. Hamayne](image)

**Molecular weight: 287.31**

**MS analysis**

EIMS \(m/z\) (%): 287 (<1), 269 (100), 240 (18), 225 (11), 224 (7), 181 (17), 115 (7), 77 (4), 56 (12), 55 (21), 54 (10), 44 (42), 43 (55), 42 (20), 41 (15)

**NMR analysis**

\(^1\text{H NMR (500 MHz, CDCl}_3\)\)
6.81 (1H, s, H-10), 6.47 (1H, s, H-7), 6.19 (2H, s, H-1, H-2), 5.90 (2H, s, OCH\(_2\)O), 4.37-4.33 (1H, m, H-3), 4.30 (1H, d, J = 16 Hz, H-6), 3.98-4.05 (1H,
m, H-11), 3.65 (1H, d, $J = 16$ Hz, H-6), 3.35 (2H, m, H-12), 3.25 (1H, dd, $J = 4.5, 3.5$ Hz, H-4a), 2.07-2.12 (2H, m, H-4)

$^{13}$C NMR (125 MHz, CDCl$_3$)

146.8, 146.3, 137.4, 135.4, 125.2, 122.9, 106.8, 103.3, 101.0, 79.5, 67.0, 65.6, 63.0, 60.5, 49.8, 33.2

Optical rotation

$[\alpha]^{25}_D = +110.9^\circ$ (c= 0.1, CHCl$_3$)

6.1.20 NB-20: Crinine

Summary formula: C$_{16}$H$_{17}$NO$_3$

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the crinine-type alkaloid crinine (Fig. 33)$^{55,195}$.

Fig. 33. Crinine

Molecular weight: 271.31

MS analysis

EIMS m/z (%): 271(100), 254(8), 242(8), 228(25), 216(14), 199(48), 187(43)

NMR analysis

$^1$H NMR (500 MHz, CDCl$_3$)

6.84 (1H, s, H-10), 6.55 (1H, d, $J = 10.2$ Hz, H-1), 6.45 (1H, s, H-7), 5.96 (1H, dd, $J = 10.2, 5.2$ Hz, H-2), 5.89 (1H, d, $J = 17.9$ Hz, overlapped, OCH$_2$O), 5.88 (1H, d, $J = 17.9$ Hz, overlapped, OCH$_2$O), 4.38 (1H, d, $J = 16.6$ Hz, H-6), 4.34 (1H, ddd, $J = 6.0, 5.2, 1.7$ Hz, H-3), 3.76 (1H, d, $J = 16.6$ Hz, H-6), 3.43-3.36 (2H, m, H-4a, H-12), 2.90 (1H, ddd, $J = 13.0, 9.0, 6.0$ Hz, H-12), 2.18 (1H, ddd,
$J = 12.2, 9.0, 4.3 \text{ Hz, H-11}$, 2.05 (1H, ddd, $J = 13.9, 4.3, 1.8 \text{ Hz, H-4}$), 1.93 (1H, ddd, $J = 12.2, 10.7, 6.0 \text{ Hz, H-11}$), 1.74 (1H, ddd, $J = 13.9, 13.6, 6.0 \text{ Hz, H-4}$)

$^{13}C$ NMR (125 MHz, CDCl$_3$)

146.3, 145.8, 138.0, 131.7, 127.7, 125.6, 106.9, 102.9, 100.8, 63.8, 62.9, 62.0, 53.4, 44.3, 43.9, 32.5

Optical rotation

$[\alpha]^{20}_D = -29.2^\circ \ (c= 0.26, \text{ CHCl}_3)$

6.1.21 NB-21: Powelline

Summary formula: C$_{17}$H$_{19}$NO$_4$

On the basis of the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the crinine-type alkaloid powelline (Fig. 34$^{154}$).

Fig. 34. Powelline

Molecular weight: 301.34

MS analysis

EIMS $m/z$ (%): 301(100), 284(8), 272(7), 258(20), 246(12), 244(12), 229(80), 217(40)

NMR analysis

$^1H$ NMR (500 MHz, CDCl$_3$)

6.57 (1H, s, H-10), 6.55 (1H, d, $J = 10.0 \text{ Hz, H-1}$), 5.95 (1H, dd, $J = 10.0, 5.0 \text{ Hz, H-2}$), 5.87 (1H, d, $J = 18.0 \text{ Hz, overlapped, OCH}_2\text{O}$), 5.86 (1H, d, $J = 18.0$...
Hz, overlapped, OCH$_2$O), 4.37-4.30 (1H, m, H-3), 4.28 (1H, d, $J = 17.5$ Hz, H-6), 3.98 (3H, s, C7-OCH$_3$), 3.85 (1H, d, $J = 17.5$ Hz, H-6), 3.43-3.38 (2H, m, H-12), 2.93-2.88 (1H, m, H-4a), 2.08-2.05 (1H, brm, H-11), 1.95-1.89 (1H, m, H-4), 1.75 (1H, dt, $J = 13.5$, 4.0 Hz, H-11)

$^{13}$C NMR (125 MHz, CDCl$_3$)

148.2, 141.0, 139.0, 133.4, 132.2, 127.4, 116.9, 100.6, 96.8, 64.0, 62.6, 59.2, 58.6, 53.7, 44.2, 43.9, 32.4,

Optical rotation

$[\alpha]_{D}^{23} = +3.9^\circ$ (c=1.0, CHCl$_3$)
6.2 Biological activity of the isolated alkaloids

6.2.1 The biological activity of the isolated alkaloids in relation to Alzheimer’s disease

Alkaloids were tested for their inhibitory activity against HuAChE, HuBuChE and POP. The values of the inhibitory activity are summarized in Table 7. The most interesting values have a yellow label. The values less than 100 μM for ChEs and 1 μM for POP are labelled in bright yellow.

Table 7. Biological activity of the isolated alkaloids

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ HuAChE (μM)</th>
<th>IC₅₀ HuBuChE (μM)</th>
<th>IC₅₀ POP (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belladine (NB-1)</td>
<td>699.2 ± 19.4</td>
<td>315.3 ± 10.5</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Undulatine (NB-2)</td>
<td>23.5 ± 1.2</td>
<td>&gt;1000</td>
<td>1.97 ± 0.12</td>
</tr>
<tr>
<td>Acetylcaranine (NB-3)</td>
<td>443.7 ± 62.4</td>
<td>141.2 ± 12.6</td>
<td>0.65 ± 0.04</td>
</tr>
<tr>
<td>Caranine (NB-4)</td>
<td>&gt;1000</td>
<td>187.6 ± 51.3</td>
<td>1.99 ± 0.33</td>
</tr>
<tr>
<td>11-O-Acetyllambelline (NB-5)</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;0.79</td>
</tr>
<tr>
<td>Buphandrine (NB-6)</td>
<td>72.6 ± 8.2</td>
<td>&gt;1000</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td>Buphanisine (NB-7)</td>
<td>99.2 ± 4.2</td>
<td>&gt;1000</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Ambelline (NB-8)</td>
<td>169.2 ± 7.4</td>
<td>985.4 ± 25.6</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Buphanamine (NB-9)</td>
<td>236.5 ± 32.3</td>
<td>626.2 ± 67.9</td>
<td>3.11 ± 0.36</td>
</tr>
<tr>
<td>4'-O-Demethylbelladine (NB-10)</td>
<td>606.8 ± 74.2</td>
<td>30.7 ± 4.0</td>
<td>0.37 ± 0.03</td>
</tr>
<tr>
<td>6-O-Demethylbelladine (NB-11)</td>
<td>223.2 ± 23.6</td>
<td>115.7 ± 10.1</td>
<td>0.66 ± 0.09</td>
</tr>
<tr>
<td>Tazettine (NB-12)</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;100</td>
</tr>
<tr>
<td>1,2β-Epoxyambelline (NB-13)</td>
<td>nm b</td>
<td>nm b</td>
<td>nm b</td>
</tr>
<tr>
<td>6α-Hydroxyundulatine (NB-14)</td>
<td>&gt;1000</td>
<td>624.8 ± 95.0</td>
<td>nm b</td>
</tr>
<tr>
<td>1-O-Acetylbulisine (NB-15)</td>
<td>84.8 ± 11.0</td>
<td>481.7 ± 84.1</td>
<td>2.45 ± 0.21</td>
</tr>
<tr>
<td>Haemantamine (NB-16)</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;100</td>
</tr>
<tr>
<td>1-O-Acetyllycorine (NB-17)</td>
<td>&gt;1000 f</td>
<td>176.2 ± 14.2</td>
<td>0.45 ± 0.05</td>
</tr>
<tr>
<td>Crinamidine (NB-18)</td>
<td>230.1 ± 9.8</td>
<td>&gt;1000</td>
<td>0.79 ± 0.06</td>
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<tr>
<td>Hamayne (NB-19)</td>
<td>992.7 ± 220.7</td>
<td>472.0 ± 37.0</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Crinine (NB-20)</td>
<td>&gt;1000</td>
<td>770.0 ± 46.9</td>
<td>1.47 ± 0.12</td>
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<tr>
<td>Powelline (NB-21)</td>
<td>29.1 ± 1.6</td>
<td>394.0 ± 4.8</td>
<td>0.77 ± 0.02</td>
</tr>
<tr>
<td>Galanthamine a</td>
<td>1.7 ± 0.1</td>
<td>42.3 ± 1.3</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Berberine c</td>
<td>-</td>
<td>-</td>
<td>0.14 ± 0.02</td>
</tr>
</tbody>
</table>

*a Results are the mean values ± standard deviations of three independent replications; b nm: not measured due to limited material; c Standard; d In higher concentrations turbidity was formed and so the IC₅₀ value could not be accurately determined; e The value of IC₅₀ in HuAChE assay; f The value of IC₅₀ in AChE assay with the use of commercially available acetylcholinesterase from electric eel.

Contrary to previous reports (IC₅₀ = 0.96 ± 0.04 mM – identical results in both reports), we found 1-O-acetyllycorine to be inactive in the HuAChE inhibition
assay (IC$_{50}$ > 1000 mM). Based on the conflicting results, we decided to perform the same experiments with commercially available acetylcholinesterase from electric eel to compare the IC$_{50}$ value of 1-O-acetyllycorine with the reported data. In these experiments (six replications), 1-O-acetyllycorine showed moderate inhibitory activity with an IC$_{50}$ value of 28.4 ± 0.35 mM. From the results obtained, it could be concluded that 1-O-acetyllycorine acts via a mixed inhibition mechanism.

The kinetic analysis of AChE inhibition is shown in Fig. 35. The $K_m$ and $V_m$ values were calculated from the Lineweaver-Burk plot$^{205}$. The values of $K_m$ and $V_m$ for reaction in the presence of 1-O-acetyllycorine were decreased compared with the values for the reaction in its absence$^{200}$.

![Lineweaver-Burk plot](image)

Fig. 35. Lineweaver-Burk plot of $1/v$ vs. $1/[acetylthiocholine]$ in the presence or absence of 1-O-acetyllycorine
6.2.2 **Cytotoxic activity against two cancer cell lines and one noncancerous gastrointestinal cell lines**

Twelve of the isolated alkaloids were tested for their cytotoxic activity against p53-mutated Caco-2 and HT-29 colorectal adenocarcinoma cells\(^{200,207}\). Healthy small intestine cells were used to determine overall toxicity against noncancerous cells. The acquired data are summarized in Table 8. The most interesting values are labelled yellow.

Table 8. Cytotoxic activity of the chosen isolated alkaloids

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Cancer cells</th>
<th>Normal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Caco-2</td>
<td>HT-29</td>
</tr>
<tr>
<td>Acetylcaranine (NB-3)</td>
<td>29.5 ± 0.6</td>
<td>19.2 ± 1.2</td>
</tr>
<tr>
<td>Caranine (NB-4)</td>
<td>64.4 ± 4.5</td>
<td>46.6 ± 1.9</td>
</tr>
<tr>
<td>11-O-Acetylamelline (NB-5)</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Buphanisine (NB-7)</td>
<td>8.6 ± 0.2</td>
<td>5.3 ± 1.7</td>
</tr>
<tr>
<td>Crinine (NB-20)</td>
<td>64.5 ± 17.8</td>
<td>50.8 ± 1.4</td>
</tr>
<tr>
<td>Haemanthamine (NB-16)</td>
<td>0.99 ± 0.14</td>
<td>0.6 ± 0.01</td>
</tr>
<tr>
<td>Hamayne (NB-19)</td>
<td>17.2 ± 0.9</td>
<td>12.4 ± 0.3</td>
</tr>
<tr>
<td>Ambelline (NB-8)</td>
<td>74.1 ± 1.1</td>
<td>50.2 ± 1.2</td>
</tr>
<tr>
<td>Undulatine (NB-2)</td>
<td>51.7 ± 1.1</td>
<td>53.4 ± 2.2</td>
</tr>
<tr>
<td>Buphanamine (NB-9)</td>
<td>53.5 ± 0.7</td>
<td>47.6 ± 2.2</td>
</tr>
<tr>
<td>1-O-Acetylbulbisine (NB-15)</td>
<td>33.4 ± 2.9</td>
<td>47.9 ± 1.6</td>
</tr>
<tr>
<td>Tazettine (NB-12)</td>
<td>22.8 ± 3.3</td>
<td>23.4 ± 2.0</td>
</tr>
<tr>
<td>Vinorelbine</td>
<td>0.03 ± 0.0</td>
<td>NT</td>
</tr>
</tbody>
</table>

\(^a\) Results are the mean values ± standard deviation of three independent replications; NT – Not Tested

6.2.3 **Antiproliferative activity of alkaloids determined by WST-1 assay**

To investigate the antiproliferative activities of the evaluated Amaryllidaceae alkaloids, the cytotoxic effect on cell survival using a single-dose primary screen was determined. Single-dose testing of growth inhibition in the screening panel of 17 human cell lines was performed with 12 different alkaloids isolated from *Nerine bowdenii* Watson at a concentration of 10 mM. At the end of the incubation period (48 h), cell proliferation was determined by WST-1 proliferation assay and related to the proliferation of DMSO vehicle treated control cells (designated as 100% proliferation). The one-dose data of all the screened compounds for all 17 human cell lines are presented in Fig. 36 and Table 9\(^{206}\).
Table 9. Values represent cell proliferation after selected alkaloid treatment and are expressed as percent of proliferation of untreated control cells. Each value is a mean of three independent experiments. Values from the intervals 0-25%, 26-50% and 50-75% are highlighted with different colours. The DMSO indicates negative control of the experiment (0.1% DMSO solvent control). Doxorubicin was used as a reference drug.

<table>
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</table>

Cell line abbreviations are as follows: HL-60 human promyelocytic leukemia; Jurkat human lymphoblast; MOLT-4 human T lymphoma; A549 human lung carcinoma; H1299 human lung adenocarcinoma; COLO-201 human colon cancer; HT-29 human colon adenocarcinoma; SW-480 human colon adenocarcinoma; A2780 human ovarian carcinoma; HeLa human cervical adenocarcinoma; BT-549 human breast cancer; MCF-7 human breast cancer; MDA-MB-231 human breast adenocarcinoma; SAOS human osteosarcoma; NHDF human dermal fibroblast.
Fig. 36. Cell proliferation after selected alkaloid treatment for all 17 human cell lines.

Each graph represents proliferation of 17 cell lines after culture with indicated alkaloid. Values were calculated as mean of three independent experiments and are
expressed as percent of proliferation of untreated control cell lines (100%). Numbers 1-17 define appropriate cell line (1. HL-60, 2. Jurkat, 3. MOLT-4, 4. A549, 5. H1299, 6. COLO-201, 7. HT-29, 8. SW-480, 9. AGS, 10. PANC-1, 11. A2780, 12. HeLa, 13. BT-549, 14. MCF-7, 15. MDA-MB-231, 16. SAOS-2, 17. NHDF). The line borders 50% value. The DMSO indicates negative control of the experiment (0.1% DMSO solvent control). Doxorubicin was used as a reference drug. Error bars indicate ±SD.

Based on the promising results obtained with haemanthamine in the single-dosed screening experiment, follow-up studies were performed to establish the alkaloid concentration which induced a 50% inhibition of cell proliferation (IC$_{50}$ values). The proliferations of evaluated cells were determined 48 h after treatment in a broad concentration range of 0.01, 0.1, 1, 10 and 100 μM. The IC$_{50}$ values of haemanthamine against the panel of 17 human cell lines are summarized in Table 10.

Table 10. IC$_{50}$ values of haemanthamine against human cancer and noncancerous cells$^{a,b}$

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Haemanthamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Jurkat</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>A549</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>H1299</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>COLO-201</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>HT-29</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>SW-480</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>AGS</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>PANC-1</td>
<td>9.8 ± 0.4</td>
</tr>
<tr>
<td>A2780</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>HeLa</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>BT-549</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>MCF-7</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>9.5 ± 0.2</td>
</tr>
<tr>
<td>SAOS-2</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>NHDF</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

$^a$ Results are expressed in μM; $^b$ Result are the mean values ± standard deviations of three independent replications
7 DISCUSSION

The Amaryllidaceae alkaloids were introduced as compounds with great potential in the research for new drugs for use in the therapy of different chronic diseases. Nowadays, the research of the mentioned compounds is targeted to their use in the therapy of cancer, especially due to their highly selective effect on the cancer cells and relatively easy isolation from the plant material. Attention is also paid to the study of the inhibitory activity against human cholinesterases, prolyl oligopeptidase and GSK-3β.

*Nerine bowdenii* Watson was chosen for closer study of its biological activity. The cholinesterase activity of the plant extract had been determined within the screening study of plants from the Amaryllidaceae family\(^5\)\(^6\)\(^7\). The inhibitory activity against human cholinesterases was determined *in vitro* by a modified Ellman’s spectrophotometric method\(^190\). The values of the inhibitory activity against HuAChE and HuBuChE of the alkaloidal extract of the chosen plant material were IC\(_{50}\) HuAChE 87.9 ± 3.5 μg/mL and IC\(_{50}\) HuBuChE 14.8 ± 1.1 μg/mL. Although, the activity against HuAChE is not as promising as that of the usually used standards (galanthamine, huperzine A), the activity against HuBuChE was very interesting and received our attention.

In order to identify the compounds of the summary extract, we used capillary GS/MS analysis. This analysis showed the presence of more than 20 alkaloids, some of which were identified based on their retention times and mass spectra. The others cannot be identified unless isolated and subjected to further analysis.

Extensive chromatographic purification of the concentrated alkaloidal extract prepared from 10 kg of fresh bulbs of *Nerine bowdenii* led to the isolation of two new and nineteen known Amaryllidaceae alkaloids. The structures of the known alkaloids were determined by comparison of their MS and NMR spectra with literature data and additional physical properties. The isolated alkaloids belong to the following structural types: belladine (belladine, 4'-O-demethylbelladine, 6-O-demethylbelladine), crinine (undulatine, 11-O-acetylamelline, buphandrine, buphanisine, ambelline, buphanamine, 1,2β-epoxyambelline, 6α-hydroxyundulatine, 1-O-acetylbulbisine, crinamidine, crinine, powelline), haemanthamine (heamanthamine, hamayne), lycorine (acetyllcaranine,
caranine, 1-O-acetyllycorine) and tazettine (tazettine). 6-O-Demethylbelladine and 4′-O-demethylbelladine were isolated for the first time from this plant. The novel compounds were obtained as a light yellow viscous mass. ESI-HRMS of these alkaloids showed molecular ion peaks \([M + H]^+\) at \(m/z\) 302.1751, and \(m/z\) 302.1750, respectively, corresponding to the formula \(C_{18}H_{23}NO_3\) (calc. 301.1678). NMR spectra of both new belladine-derivatives are similar to those of belladine\(^{126}\). They differ only by the absence of one methoxy group in the spectra of both compounds (the signal of this group is missing in the \(^1\)H and \(^13\)C NMR spectra). Assignments of the chemical shifts in both compounds were made by employing gHSQC and gHMBC experiments. In the case of 4′-O-demethylbelladine, there is correlation between the hydrogens of the OCH\(_3\) group and carbon C6 (δ = 158.3 ppm). Further correlation in the gHMBC experiment indicates the presence of a second methoxy group at carbon C5′ (δ = 146.9 ppm). Thus, carbon C4′ bears only a hydroxy group. In the case of 6-O-demethylbelladine, gHMBC experiment indicates correlations between hydrogens of the methoxy groups and carbons C4′ (δ = 149.1 ppm) and C5′ (δ = 148.7 ppm) leading to the conclusion that the methoxy group is missing at carbon C6.

All of the isolated compounds obtained in sufficient amounts were assayed for various biological activities. The inhibitory activities against HuAChE and HuBuChE of the isolated alkaloids and their potential use in the therapy of AD are subjects of long-term study at the Department of Pharmaceutical Botany. The importance of the cholinesterases is mentioned in the chapter Biological activity of the Amaryllidaceae alkaloids in the Theoretical part. The most active alkaloids in the HuAChE assay were two crinine-type alkaloids, undulatine and powelline, with IC\(_{50}\) values of 23.5 ± 1.2 μM and 29.1 ± 1.6 μM. Previously, undulatine has been reported as an important AChE inhibitor, which acts via a mixed inhibition mechanism and, on the basis of the PAMPA and BBB method, is able to cross the BBB by passive permeation. Additionally, undulatine has shown interesting POP inhibitory activity\(^{54,208}\). Contrary to the previous reports (IC\(_{50}\) 0.96 ± 0.04 μM – identical results in both reports)\(^{203,204}\), we found 1-O-acetyllycorine to be inactive in the HuAChE inhibition assay (IC\(_{50}\) > 1000 μM). Based on the conflicting results, we decided to perform the same experiments with commercially available acetylcholinesterase from electric eel to compare the IC\(_{50}\) value of 1-O-acetyllycorine with reported data. In these experiments (six replications), 1-O-
acetyllycorine showed moderate inhibitory activity with an IC\textsubscript{50} value of 28.4 ± 0.35 μM. It seems that the source of enzyme is, in some cases, crucial for the determination of AChE inhibitory activity. For the determination of the inhibition mechanism of 1-\textit{O-}acetyllycorine, a Lineweaver-Burk plot was used\textsuperscript{203}. In the BuChE inhibition assay we obtained similar data for each type of BuChE (IC\textsubscript{50} 176.2 ± 14.2 μM for HuBuChE and IC\textsubscript{50} 93.6 ± 5.2 μM for horse serum BuChE). The most potent HuBuChE inhibition activity has been demonstrated by the newly identified alkaloid 4'-\textit{O-}demethylbelladine (IC\textsubscript{50} 30.7 ± 4.0 μM). The other tested alkaloids showed only weak or no inhibition activity in the HuAChE and HuBuChE assays. The compounds were also tested for their POP inhibitory activity. This cytosolic serine peptidase plays a role in the biosynthesis and degradation of peptide hormones and neuropeptides present in the brain. They are involved in the process of learning and memory. The real effect and role of POP in AD has not been fully determined yet, but it is considered that its inhibition could lead to the improvement of cognitive functions\textsuperscript{210}. The inhibition of POP can represent an important supporting approach in AD treatment. We tested all isolated compounds for their ability to inhibit POP. The most interesting inhibition activity has been demonstrated by 4'-\textit{O-}demethylbelladine (belladine type), buphandrine (crinine type) and 1-\textit{O-}acetyllycorine (lycorine type) with IC\textsubscript{50} values of 0.37 ± 0.03 mM, 0.37 ± 0.04 mM and 0.45 ± 0.05 mM. Some of the Amaryllidaceae alkaloids have been previously tested for their POP inhibition activity. In comparison with the used standard, an isoquinoline alkaloid, berberine, the best obtained activities were about three times weaker. However, only a limited number of Amaryllidaceae alkaloids have been tested so far. The lycorine structure seems to be interesting for POP inhibition, but a wider range of compounds of either natural origin of semisynthetic analogues must be tested first.

Nowadays, attention is also being paid to the inhibition of GSK-3\textbeta. This enzyme plays a key role in diverse physiological processes including metabolism, the cell cycle, and gene expression by regulating a wide variety of well-known substances like glycogen synthase, tau-protein, and β-catenin. Recent studies have identified GSK-3\textbeta as a potential therapeutic target in Alzheimer’s disease, bipolar disorder, stroke, more than 15 types of cancer, and diabetes. In the case of \textit{Nerine bowdenii}, a lycorine-type
alkaloid, caranine, was the most potent inhibitor of GSK-3β, with an IC$_{50}$ value of 30.75 ± 0.04 μM (SB-415286 used as a standard, IC$_{50} = 70.00$ nM)$^{211}$.

The potential usage of Amaryllidaceae alkaloids in the treatment of cancer has been mentioned in the theoretical part of this work. The big advantage of these compounds is their selectivity towards the cancer cells and low toxicity for the non-cancerous cells. Twelve alkaloids (undulatine, haemanthamine, hamayne, ambelline, 1-$O$-acetylbulbisine, buphanamine, tazettine, acetylcara nine, caranine, 11-$O$-acetylambelline, buphanisine and crinine) isolated from Nerine bowdenii have been tested for their cytotoxicity against p53-mutated Caco-2 and HT-29 colorectal adenocarcinomas cells. At the same time, healthy small intestine cells, Fhs-74 Int, were used to determine overall toxicity against non-cancerous cells. From the tested compounds, interesting cytotoxicity has been shown by the α-crinine-type alkaloid buphanisine, which showed an interesting toxicity against both p53-mutated Caco-2 and HT-29 colorectal adenocarcinoma cells (IC$_{50}$ Caco-2 8.6 ± 0.2 μM, IC$_{50}$ HT-29 5.3 ± 1.7 μM), while showing significantly lower toxicity against normal intestine Fhs-74 Int cells (IC$_{50}$ 22.8 ± 2.6 μM). The basic SAR of crinine-type alkaloids can be hypothesized from the study of McNulty$^{209}$, who screened a mini-library of crinane-type alkaloids for their ability to induce apoptosis in rat liver hepatoma (5123c) cells. The potent cytotoxic activity of crinine-type alkaloids is connected with the presence of α-C2 bridge, such as in haemanthamine and haemanthidine$^{207}$. Alkaloids with a β-C2 bridge in their structure, such as in the tested buphanisine, showed no cytotoxicity except for a rare alkaloid, isolated from Boophane disticha, which demonstrated significant antiproliferative activity in human acute lymphoblastic leukemia (CEM) cells$^{197}$.

In conclusion, we can state that Nerine bowdenii is a rich source of diverse Amaryllidaceae alkaloids, especially of the crinine type, with important biological activities. Compounds isolated in sufficient amount were screened for their biological activities connected with Alzheimer’s disease and oncological diseases. Some alkaloids were isolated in amounts that will allow detailed study of their mechanism of action and also preparation of new derivatives for biological assays.
8 ABSTRACT

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Department of Pharmaceutical Botany
Candidate: **Mgr. Nina Vančeková**
Supervisor: **Doc. Ing. Lucie Cahlíková, PhD.**
Title of Doctoral Thesis: **Study of the inhibitory (toxic) effect of the alkaloids from chosen plants of Amaryllidaceae family on some human enzymatic systems (in vitro study) II.**

Key words: *Nerine bowdenii*, Amaryllidaceae, alkaloids, biological activity, acetylcholinesterase, butyrylcholinesterase, prolyl oligopeptidase, cytotoxicity

An alkaloid extract prepared from the fresh bulbs of *Nerine bowdenii* Watson was chosen as a source of a variety of Amaryllidaceae alkaloids with a potential for interesting biological activity. The mixture of alkaloids, prepared by standard extraction techniques, was fractionated by column chromatography on aluminium oxide using step gradient elution with petrol, chloroform and ethanol. The isolation of 21 alkaloids was a result of column chromatography, preparative TLC and crystallizations. Two of these alkaloids were isolated from this plant for the first time. The chemical structures of the isolated compounds were determined on the basis of spectrometric techniques (NMR, MS, optical rotation) and by comparison with literature data. If the amount of the isolated alkaloid were sufficient, it was tested for its biological activity, in particular inhibitory activity against human cholinesterases and prolyl oligopeptidase, cytotoxicity and inhibition of GSK-3β.

The inhibitory activity against human cholinesterases was determined in vitro by a modified Ellman’s spectrophotometric method. Undulatine (NB-2) and powelline (NB-21) were the most potent inhibitors of AChE with IC₅₀ values of 23.5 ± 1.2 μM for undulatine and 29.1 ± 1.6 μM for powelline. In the context of inhibitory activity against BuChE, most of the isolated alkaloids proved inactive (IC₅₀ >100 μM). The only exception was 4'-O-demethylbelladine with a value of IC₅₀ 30.7 ± 4.0 μM.

The POP inhibition activity was determined using a spectrophotometric method
with Z-Gly-Pro-\(p\)-nitroanilid as a substrate. The IC\(_{50}\) values of acetylcaranine, buphandrine, 4'-O-demethylbelladine, 6-O-demethylbelladine, 1-O-acetyllycorine, crinamidine, and powelline were under 1 \(\mu\)M. The activity closest to that of the standard berberine was shown by buphandrine (IC\(_{50}\) 0.37 ± 0.04 \(\mu\)M) and 4'-O-demethylbelladine (IC\(_{50}\) 0.37 ± 0.03 \(\mu\)M).

From all of the isolated alkaloids that were tested for their cytotoxicity, only haemanthamine and buphanisine demonstrated significant activity. The tested cell lines were Caco-2, HT-29 and healthy cell lines FHs 74 Int. The IC\(_{50}\) values for the mentioned alkaloids and cell lines were: haemanthamine – Caco-2 IC\(_{50}\) 0.99 ± 0.14 \(\mu\)M; HT-29 IC\(_{50}\) 0.6 ± 0.01 \(\mu\)M; FHs 74 Int IC\(_{50}\) 19.5 ± 8.9 \(\mu\)M and buphanisine – Caco-2 IC\(_{50}\) 8.6 ± 0.2 \(\mu\)M; HT-29 IC\(_{50}\) 5.3 ± 1.7 \(\mu\)M; FHs 74 Int IC\(_{50}\) 22.8 ± 2.6 \(\mu\)M.
9 ABSTRAKT

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Katedra farmaceutické botaniky
Kandidát: Mgr. Nina Vaněčková
Školitel: Doc. Ing. Lucie Cahlíková, Ph.D.

Název práce: Studium inhibičního (toxického) vlivu alkaloidů vybraných druhů rostlin z čeledi Amaryllidaceae na některé lidské enzymové systémy (in vitro studie) II.

Klíčová slova: Nerine bowdenii, Amaryllidaceae, alkaloidy, biologická aktivita, acetylcholinesteráza, butyrylcholinesteráza, prolyloligopeptidáza, cytotoxicita

Alkaloidní extrakt, připraven z čerstvých cibulí, rostliny Nerine bowdenii Watson byl vybrán jako zdroj různých strukturních typů Amaryllidaceae alkaloidů s potenciálně zajímavou biologickou aktivitou. Směs alkaloidů, připravená pomocí základních metod extrakce, byla dělena za použití sloupcové chromatografie. Jako stacionární fáze byl použit oxid hlinitý. Benzín, chloroform a etanol byly použity jako mobilní fáze. Koncentrace jednotlivých mobilních fází se na základě postupné gradientové eluce měnila. Výsledkem sloupcové chromatografie, preparativní TLC a krystalizace byla izolace 21 alkaloidů, z nichž dva byly z této rostliny izolovány poprvé. Chemické struktury izolovaných sloučenin byly stanoveny na základě spektrometrických technik (NMR, MS, optická rotace) a porovnáním s daty uvedenými v literatuře. Pokud byl alkaloid izolován v dostatečné množství, byly provedeny testy na stanovení jeho biologické aktivity, konkrétně inhibiční aktivita vůči acetyl-, butyrylcholinesteráze a prolyloligopeptidáze, cytotoxicita a inhibice GSK-3β.

Inhibiční aktivita vůči lidským cholinesterázám byla určena pomocí in vitro modifikované Ellmanovy spektrofotometrické metody. Nejvyšší inhibiční aktivitu vůči AChE vykazovaly undulatin (NB-2) s hodnotou IC₅₀ 23,5 ± 1,2 µM a powellin (NB-21), u kterého byla naměřena hodnota IC₅₀ 29,1 ± 1,6 µM. V případě inhibiční aktivity vůči BuChE se většina alkaloidů jevila jako neaktivní (IC₅₀ >100 µM). Jedinou výjimkou byl 4’-O-demethylbelladin jehož hodnota IC₅₀ byla 30,7 ± 4,0 µM.
Inhibiční aktivita vůči POP byla měřena pomocí spektrofotometrické metody za použití Z-Gly-Pro-p-nitroanilidu jako substrátu. Hodnoty IC\textsubscript{50} naměřené u alkaloidů acetylkaraninu, buhandrinu, 4’-O-demetyllbelladinu, 6-O-demetyllbelladinu, 1-O-acetylllykorinu, krinamidinu, powellinu byly nižší než 1 µM. Nejvyšší aktivita byla naměřena u buhandrinu (IC\textsubscript{50} 0,37 ± 0,04 µM) a 4’-O-demetyllbelladinu (IC\textsubscript{50} 0,37 ± 0,03 µM), jejichž hodnoty byly nejblíž hodnotám naměřených u standardu berberinu.

Ze všech izolovaných alkaloidů, které byly testovány na cytotoxicitu se pouze haemanthamin a buphansin projevily jako významně aktivní. Testovanými nádorovými buněčnými liniemi byly Caco-2 a HT-29 a zdravé buněčné linie FHs 74 Int. Hodnoty IC\textsubscript{50} zmíněných alkaloidů u jednotlivých buněčných linií byly: u haemanthaminu – Caco-2 IC\textsubscript{50} 0,99 ± 0,14 µM; HT-29 IC\textsubscript{50} 0,6 ± 0,01 µM; Fhs-74 Int IC\textsubscript{50} 19,5 ± 8,9 µM a u buphansinu – Caco-2 IC\textsubscript{50} 8,6 ± 0,2 µM; HT-29 IC\textsubscript{50} 5,3 ± 1,7 µM; Fhs 74 Int 22,8 ± 2,6 µM.
10 COMPENDIUM OF PUBLICATIONS

10.1 Publications


Cahlíková L., Hrabinová M., Kulhánková A., Benešová N., Chlebek J., Jun D., Novák Z., Macáková K., Kuneš J., Kuča K., Opletal L.: Alkaloids from *Chlidanthus fragrans* and their acetylcholinesterase, butyrylcholinesterase and prolyl oligopeptidase activities. *Natural Product Communications*, 2013, 8, 1541-1544. (IF_{2017}=0.809)

Cahlíková L., Ločárek M., Benešová N., Kučera R., Chlebek J., Novák Z., Opletal L.: Isolation and cholinesterase inhibitory activity of *Narcissus* extracts and
amaryllidaceae alkaloid. *Natural Product Communications*, 2013, 8, 781-785. (IF$_{2017}$=0.809)


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**10.2 Monography**


**10.3 Conferences**

**10.3.1 Lectures**

Benešová N., Cahlíková L., Novák Z., Opletal L.: Alkaloids of *Nerine bowdenii* Watson (Amaryllidaceae) and their biological activity. 3$^{rd}$ Postgraduate and 1$^{st}$ Postdoctoral Science Conference, Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Czech Republic, 28.-29.1.2013

Benešová N., Opletal L., Novák Z., Cahlíková L.: Alkaloids of *Nerine bowdenii* Watson (Amaryllidaceae) and their biological activity. 4$^{th}$ Postgraduate and 2$^{nd}$ Postdoctoral Science Conference, Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Czech Republic, 28.-29.1.2014

**10.3.2 Posters**

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