FACULTY OF PHARMACY
IN HRADEC KRÁLOVÉ
Charles University

The Dissertation

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Alkaloids of the genus *Hippeastrum* (Amaryllidaceae): isolation, identification, biological activity

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Branch of study: Pharmacognosy and Nutraceuticals

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 acknowledgment is made in the text of the Thesis.

Hradec Králové, April 2021 MSc. Latifah Al Shammari

Acknowledgments

All praises for the most omnipotent, omnipresent, and omniscient Almighty Allah, who blessed me to accomplish my doctoral research work. All respect for his last Holy Prophet (Peace be upon Him), whose teaching inspired me to wider my thoughts and deliberate things deeply.

My most sincere thanks go to my supervisor, Prof. Ing. Lucie Cahlíková, Ph.D., for giving me this valuable chance to work in her group and for the excellent work facilities at the Department of Pharmaceutical Botany, I thank her for introducing me to the wonders and frustrations of scientific research. I thank her for her guidance, suggesting the research project, encouragement and support during every stage of my research work, and her valuable suggestions in both work and life issues.

I am grateful for the funding sources that allowed me to pursue my Ph.D. research studies, the Specific scientific research of Charles University, Faculty of Pharmacy in Hradec Králové, Nr. SVV UK 260 412; 260 401; Progres/UK Q40 and Q42), and by the Pre-application, research into innovative medicines and medical technologies project (Reg. No. CZ.02.1.01/0.0/0.0/18_069/0010046) co-funded by the European Union that allow me to complete my postgraduate studies.

Any sense of gratitude will be incomplete without giving glory and words of thanks to Dr. Daniela Hulcová for her guidance and assistance. For her willingness to help and give her time for cooperation and best suggestions, without her efforts my job would have undoubtedly been more difficult.

I would like to express my gratitude and appreciation to the whole ADINACO group, especially Prof. RNDr. Lubomír Opletal, Ph.D. for his valuable advice and wisdom. My sincere appreciation is extended to my colleagues in the Department of Pharmaceutical Botany for providing me valuable assistance, moral support, and smiling moments to overcome the difficulties.

Every result described in this thesis was accomplished with the help and support of other departments and universities. My great appreciation to all of them. Especially Doc. PharmDr. Jiří Kuneš, Ph.D. (measurements and interpretations of the NMR spectra of the alkaloids), Doc. PharmDr. Daniel Jun, Ph.D. and Mgr. Martina Hrabinová (determination of the inhibitory POP activity), RNDr. Radim Havelek, Ph.D. and Ing. Ivo Doskočil (measurements of the cytotoxic activity).

Words cannot adequately express my gratitude towards my husband, Faiz Al Shammary, who endured many sacrifices during my graduate years, without his love and endless support I would not have been able to accomplish this dissertation. I am grateful to my daughters, Khlood and Rawan for filling my life with joy and motivation. Furthermore, I wish to thank my sister; Aftah for her smart and valuable advice and the rest of my family for their support and encouragement.

In the end, I would like to thank you for your financial support. This project and thesis have been supported by various Charles University grants (SVV UK 260 412; 260 401; Progres/UK Q40 and Q42), and by the Pre-application, research into innovative medicines and medical technologies project (Reg. No. CZ.02.1.01/0.0/0.0/18_069/0010046) co-funded by the European Union.

To All of You, Thank You Very Much!

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ABBREVIATIONS

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

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1 INTRODUCTION

Natural products offer a wealth of bio-structural information that can be used to guide drug discovery and molecular design projects [1]. Plants have played a huge role in traditional medicine, and plant screening has become an important tool in drug discovery for pharmaceutical companies and university institutes [2].

The history of natural products dates back practically to the emergence of human civilization [3]. Since then, natural products have played a considerable role in health protection and disease prevention. The ancient civilians of Mesopotamia, Egypt, China, Indian, and Greece provided written evidence for the use of natural sources for curing diverse diseases [4]. The usefulness of plants for treating diseases was recorded in the Emperor Shen Nung's classic herbals of China (2500 BC) and Eber's papyrus in Egypt (1550 BC). However, it was not until the 19th century that scientists isolated active components from various medicinal plants. Natural remedies play an irreplaceable part in therapy [5]. 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 [5].

Most biologically active substances of natural origin belong to the secondary metabolites, which form a very wide range of diverse structures, and the spectrum of effects [6]. One of the most important groups of secondary metabolites is alkaloids. These are organic substances that contain at least one nitrogen atom in their molecule and are derived mainly from various amino acids or from intermediates that are formed during the biosynthesis of terpenoids, steroids, some acids, and purines [7]. The important families containing alkaloids are Papaveraceae, Solanaceae, Berberidaceae, Rubiaceae, and last but not least the family Amaryllidaceae, which contains a characteristic group of secondary metabolites called Amaryllidaceae alkaloids (AAs).

AAs are one of the most important groups of alkaloids. The amino acids tyrosine and phenylalanine are their biosynthetic precursors. To date, over 600 alkaloids belonging to various structural types have been isolated and structurally described from plants of the Amaryllidaceae family. Many of these substances have been tested for a variety of effects [8-11]. The alkaloids of the Amaryllidaceae family are significant and biologically active. The most important biological activities are inhibition of human cholinesterases, and apoptosis-inducing anticancer activity [12; 13]. Interestingly, biological activity is often connected with certain structural types. At present, these substances are known mainly for galanthamine, which

is used clinically under the trade name Reminyl® as selective, reversible acetylcholinesterase (AChE) inhibitor in the symptomatic treatment of Alzheimer's disease (AD) [14]. The disease manifests itself clinically as dementia and is also one of the primary causes of death. Another interesting substance is lycorine, which is characterized by a wide range of biological activities, of which antitumor activity is currently being intensively studied [15]. The advantage of this substance is its selectivity for tumor cells and relatively low toxicity to representatives of healthy cells [16]. A significant advantage of lycorine is its availability from natural sources, it is the most widespread AAs, which allows the isolation in sufficient quantities for biological studies, and at the same time the preparation of various semisynthetic derivatives.

This dissertation deals with the isolation, identification, and biological activity of the alkaloids from the fresh bulbs of *Hippeastrum* x *hybridum* cv. Ferrari. This plant was chosen for a phytochemical study based on GC/MS screening studies performed on various plants from the genus *Hippeastrum* [17]. It was intended to choose a plant that would be a source of the widest spectra of alkaloids, alkaloids not yet described, and which could be isolated in amounts allowing a wide range of biological studies optionally, the preparation of the semisynthetic derivatives.

2 AIMS OF THE DISSERTATION

The aims of this dissertation thesis were the isolation and identification of the alkaloids from the concentrated alkaloidal extract prepared from the fresh bulbs of *Hippeastrum* x *hybridum* cv. Ferrari (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 *Hippeastrum* and their biological activity.
- Isolation of the wide spectrum of alkaloids from the bulb extract of *Hippeastrum* x *hybridum* cv. Ferrari.
- 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, eventually the preparation of semisynthetic derivatives for structure-activity relationship studies (SAR study).

3 THEORETICAL PART

3.1 Amaryllidaceae family: history and traditional use

Plants of the Amaryllidaceae family, containing approximately 85 genera and 1100 species, have a wide distribution through both tropical and sub-tropical regions worldwide [18]. 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.).

For centuries, Amaryllidaceae plants have been cultivated not only as ornamental plants for their colorful flowers and fragrant oils but also as folk herbal medicines against various diseases in many countries and areas [18]. 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. 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 [19].

In rural South Africa, where health care facilities are often lacking, a large sector of the community uses traditional medicines. The Southern Sotho, and Zulu tribes have used a decoction of the bulbs from *Boophane* Herb., *Brunsvigia* Heist., *Crinum* L., and *Nerine* Herb. in the treatment of cough and colds, renal, and hepatic conditions, to relieve back pain, and as a remedy for infertility [20-22]. *Boophane disticha* (L.f.) Herb. is known as a toxic plant, containing compounds with alleged hallucinogenic potential. However, bulb scales or infusions are used on septic wounds and external sores, as well as for rheumatism and relief of pain. Decoctions are also used for the treatment of headaches, cramps, and internal pains [23].

The bulbs of the genus *Brunsvigia* are applied as antiseptic dressings on fresh wounds, whereas bulb decoctions are administered for the treatment of coughs, colds as well as abdominal, renal, and liver complaints [23]. A root infusion of *Clivia miniata* (Lindl.) Regel is used to treating snakebites and wounds. Besides, its roots and leaves are taken by South African women during pregnancy and child birth [23]. One of the earliest recorded uses of the plant of genus *Crinum* is as a powerful emetic. In Asia and America, the bulbs were used to "hasten the ripening of indolent tumors," while *Crinum zeylanicum* was used in the Moluccas as a strong poison [24].

Species belonging to the genus *Cyrtanthus* are among the plants used in South African traditional medicine. *C. mackenii* is used as a protective charm against storms and evil [25]. The

genus *Zephyranthes* has been used as a folk medicine for the treatment of various diseases in different countries. *Z. parulla* and *Z. rosea* have been used in the treatment of breast cancer [26; 27]. The decoction of the leaves of *Z. candida* has been used as a remedy for diabetes mellitus in South America, and *Z. candida* in mainland China to treat infantile convulsions, epilepsy, and tetanus [28]. The overview of the traditional use of Amaryllidaceae plants is summarized in the following table (Table 1).

Table 1. Traditional use of selected Amaryllidaceae plants

Plant	Use	Country	Citation
Amaryllis belladonna L.	Treatment of cancer, flowers have antispasmodic action	South Africa	[21; 29]
Apodolirion buchananii Bak.	Treatment of stomach complaints	South Africa	[30]
Boophane disticha (L.f.) Herb.	Headaches, chest pains, bladder pains, cramp-like pains in the calf muscles	South Africa	[30]
Brunsvigia grandiflora Lindl.	Treatment of coughs, colds, renal and liver problems, and stomach complaints	South Africa	[30]
Clivia miniata (Lindl) Regel	Treatment of snakebite, facilitate delivery at childbirth and induce labor	South Africa	[21; 30-32]
Crinum amabile Donn	Emetic, rheumatism, earache	Vietnam	[24; 33]
Crinum latifolium L.	Prostate carcinoma	Vietnam	[34]
Crinum macowanii Bak.	Treatment of sexually transmitted diseases, emetic, backache and increase lactation in women	South Africa	[35]
Eucharis grandiflora Planch. & Linden	Treatment of tumors, wounds	Peru, Chile	[36]
Gethyllis linearis L.	Digestive disturbances	South Africa	[37]
Haemanthus coccineus L.	Diuretic and antiasthmatic	South Africa	[38]
Haemanthus natalensis Hook.	Emetic	South Africa	[39]
Lycoris aurea Herb.	Treatment of burns, scalds, and ulcers.	China	[40]
Lycoris radiata Herb.	Treatment of sore throats, carbuncle, cancer, suppurative wounds, poliomyelitis, mastitis, ulcer, and neurodegenerative diseases like AD	China	[41-45]
Zephyranthes rosea Lindl.	Treatment of breast cancer	China	[26; 27; 46]

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 [15].

In the last decades, the different types of extracts prepared from the Amaryllidaceae plant and their isolated alkaloidal constituents have exhibited various biological activities including antimicrobial, antimalarial, antitumor, antiparasitic, anti-inflammatory, and cholinesterases inhibition [47; 48]. However, the pharmacological importance of the family received much attention after approval of galanthamine by the FDA (Food and Drug Administration) in 2000 for the therapy of mild and severe stages of AD. Galanthamine, originally isolated from *Galanthus woronowii* Losinsk. [49], represents the first prescription drug emanating from the Amaryllidaceae plants, and it was introduced into the market in 2000 in form of its hydrobromide. Other promising molecules which are intensively studied are pancratistatin, narciclasine, lycorine, and haemanthamine which demonstrated selective anticancer activity against different cancerous cell lines [18; 50-52].

3.2 Biosynthesis of Amaryllidaceae alkaloids: Norbelladine pathway

The biosynthetic pathway of the AAs usually follows four stages, starting with the enzyme preparation of precursors from the amino acids L- phenylalanine (L-Phe) and L-tyrosine (L-Tyr). Although L-Phe and L-Tyr are closely related in chemical structure, they are not interchangeable in plants [47].

In the AAs, L-Phe serves as a primary precursor of the C_6-C_1 fragment, corresponding to ring A and the benzylic position (C-$_6$), whereas, L-Tyr is the precursor of ring C, the two-carbon side chain (C-$_{11}$ and C-$_{12}$), and nitrogen, C_6-C_2-N. The conversion of L-Phe to the C_6-C_1 unit requires the loss of two carbon atoms from the side chain as well as the introduction of at least two oxygenated substituents into the aromatic ring, which occurs through cinnamanic acid or its derivatives, involving the participation of the enzyme phenylalanine ammonia lyase (PAL). The fragmentation of the cinnamic acid involves oxidation of the *β*-carbon to the ketone or acid level, where the final product is protocatechuic aldehyde. On the other hand, L-Tyr is degraded no further than tyramine before incorporation into the Amaryllidaceae alkaloids (Fig. 1) [53]. The second stage involves merging the biosynthesis of tyramine and the protocatechuic aldehyde, resulting in norbelladine by forming a Schiff's base. This reaction occupies a pivotal position since it represents the entry of primary metabolites into a secondary metabolic pathway. Then norbelladine can undergo oxidative coupling of phenols in Amaryllidaceae plants, once ring A has been suitably protected by methylation, which is considered as the third step (Fig. 1).

Finally, the last stage includes. 4′-*O*-Methylnorbelladine undergoes three types of intramolecular oxidative couplings: *ortho-para′, para-para′*and *para-ortho′*, which leads to the formation of basic structural types of AAs (Fig. 2) [53]. The *ortho-para′*phenolic coupling gives rise to the lycorine and homolycorine types of AAs via norpluviine. A reoxidation of the carbon atom in the central nitrogen containing ring leads to ring opening, following an intramolecular rotation and hemiacetal formation, homolycorine type alkaloids are formed. Similar oxidation as described previously starts from haemanthamine, which is a direct product of a *para-para′* phenolic coupling. The oxidation results in an epimeric mixture of haemanthidine and epihaemanthidine. Tazettine is then formed by irreversible intramolecular rotation via pretazettine, a direct biosynthetic precursor of tazettine [53].

*Para-ortho′*coupling leads to the formation of pharmaceutically important alkaloid galanthamine. It is proposed that the *para-ortho′*oxidative phenol coupling of key intermediate, followed by a spontaneous closure of an ether bridge results in *N*-demethylnarwedine, which is subsequently reduced to norgalanthamine, and finally *N*-methylated to galanthamine. The

belladine-type alkaloids are thought to originate from the simple methylation of norbelladine (Fig. 2) [54].

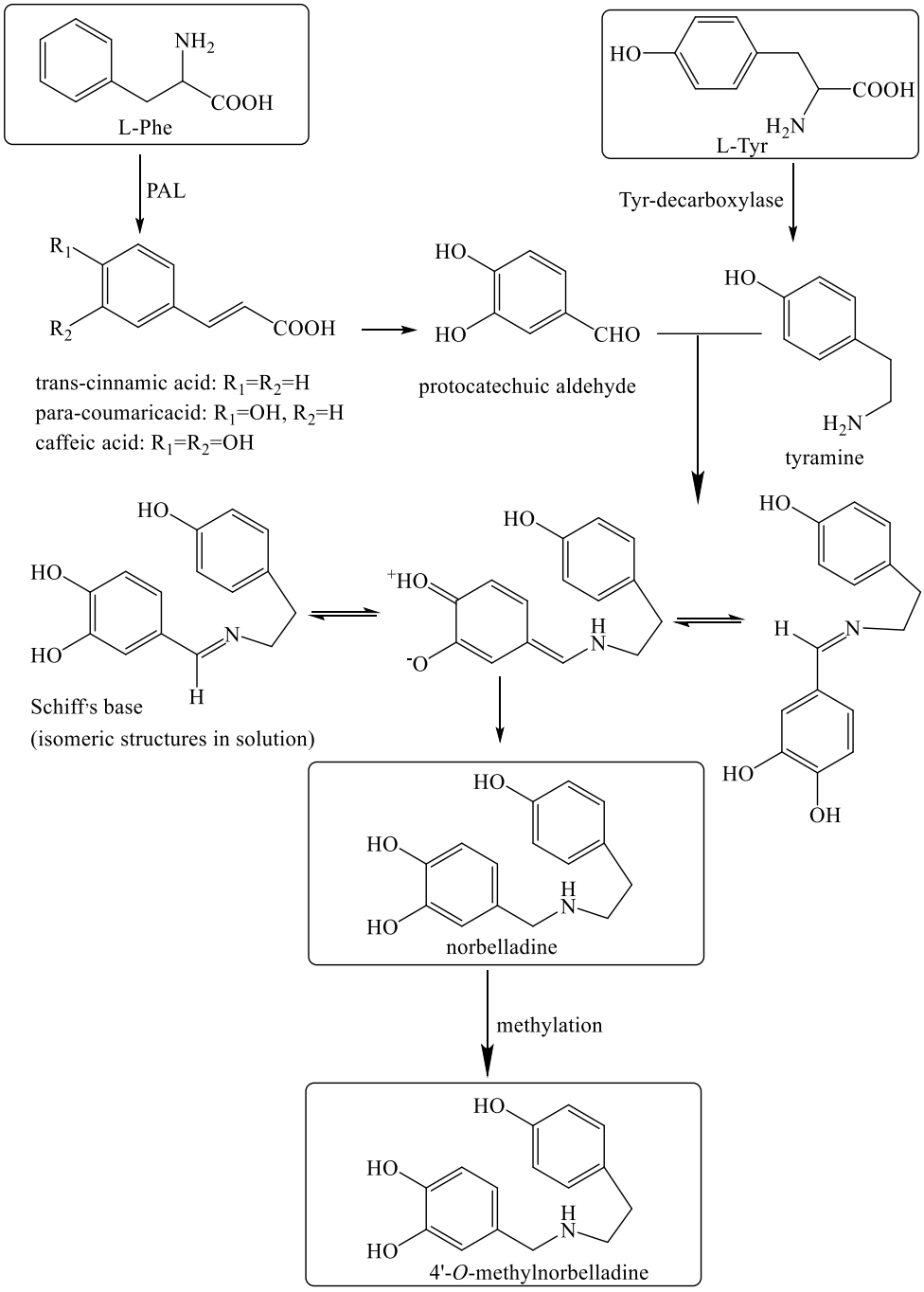


Fig. 1. Norbelladine-biosynthetic pathway to norbelladine.

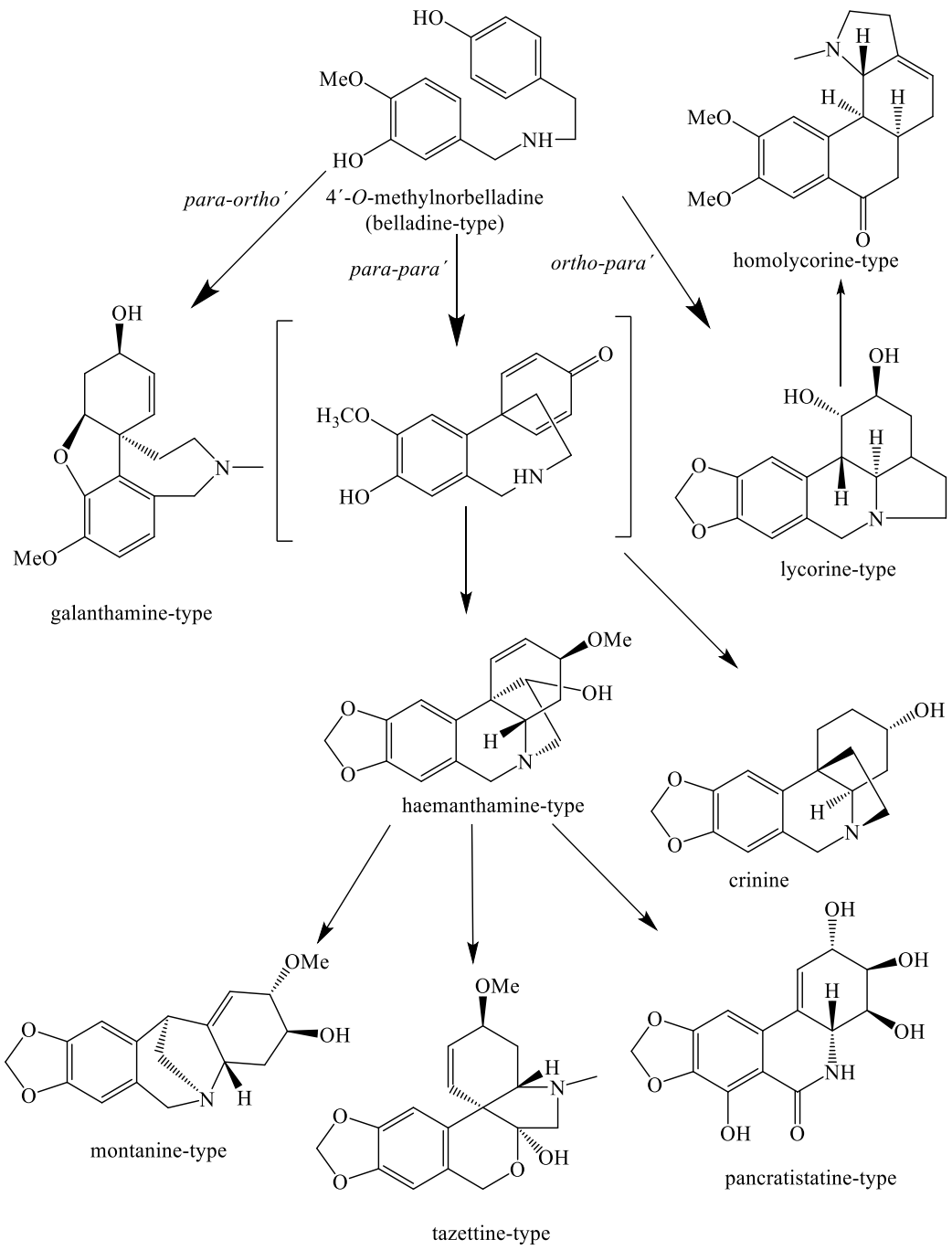


Fig. 2. Norbelladine-biosynthetic pathway: formation of the main structural types of Amaryllidaceae alkaloids

In addition to the already mentioned main structural types of AAs, several other structural types have been isolated from different Amaryllidaceae plants, whose biosynthetic pathway has not been sufficiently explained. To the best of our knowledge, twenty different structural types of AAs have been discovered. These structural types include for example galanthindole, ismine, plicamine, secoplicamine, mesembrine, and others [53; 55-59]. Structures of mentioned AAs are summarized in the following figure (Fig. 3).

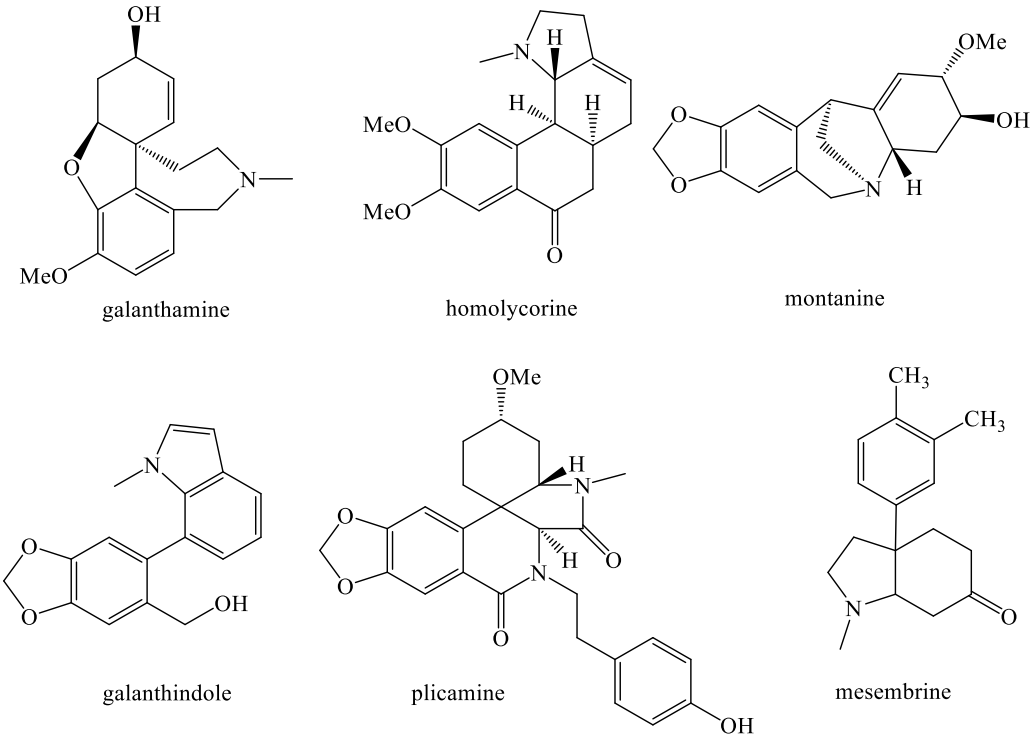


Fig. 3. Structures of selected AAs

Recently, alkaloids of two new structural types, namely narcikachnine-, and carltonine-type, have been isolated and described by our research group [60; 61]. Narcikachnine-type of AAs combines in its structure galanthamine-, and galanthindole-core, representatives of this structural-type have been isolated from fresh bulbs of *Zephyranthes citrina*, *Narcissus poeticus* cv. Pink Parasol and *Narcissus pseudonarcissus* L. cv. Dutch Master [56; 61; 62]. These new alkaloids include narciabduliine, narcipavline, narcikachnine, narcimatuline, and narcieliine (Fig. 4). Further new alkaloids carltonine A, B, and C, isolated from fresh bulbs of *Narcissus pseudonarcissus* cv. Carlton [60] are dimeric derivatives of norbelladine type with additional galanthindole-core in their structure (Fig. 5). All these new alkaloids demonstrated promising biological activities connected with the potential treatment of AD (see Fig. 4 and 5.).

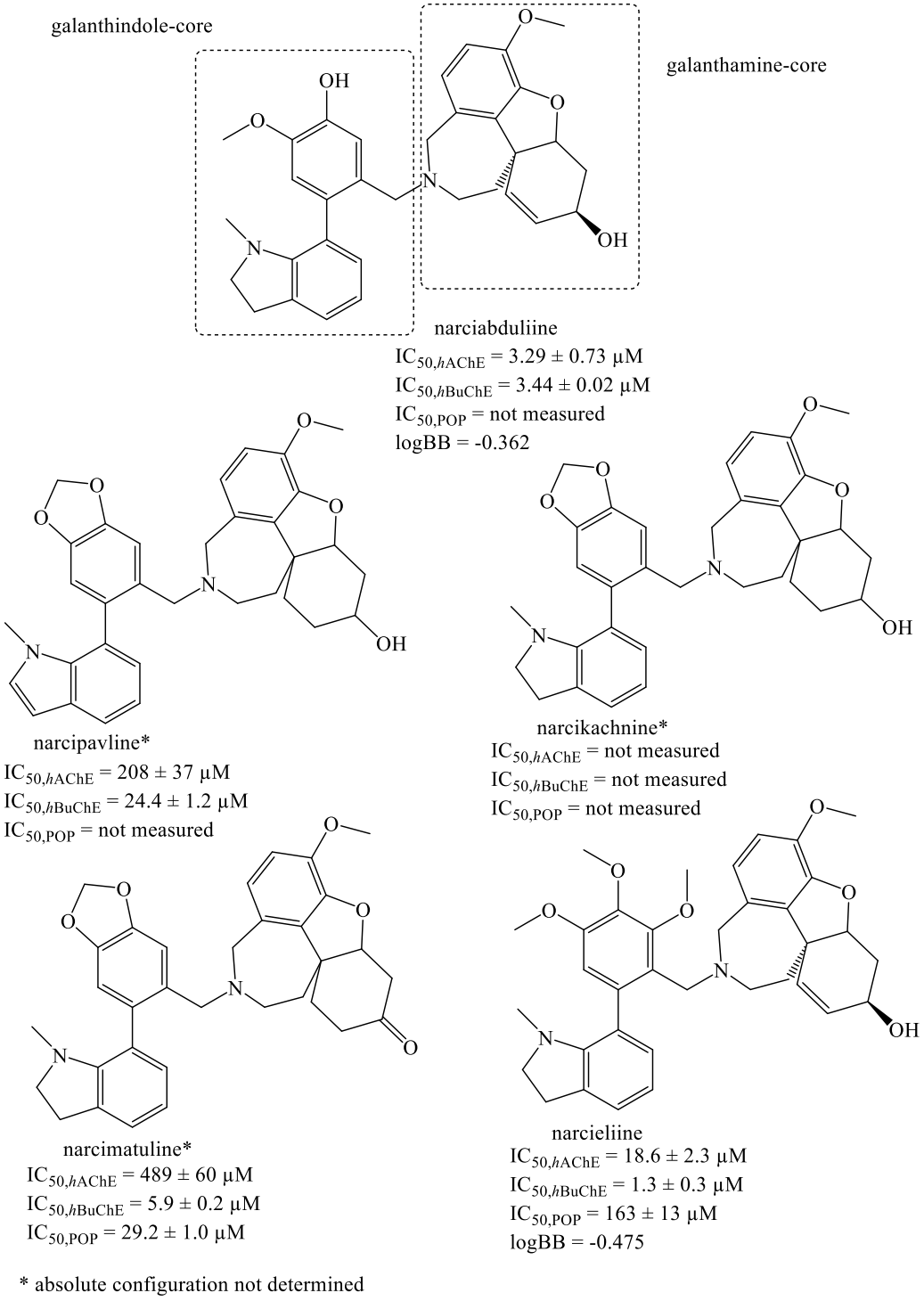


Fig. 4. Structures of narcikachnine-type AAs along with their biological activities connected with AD.

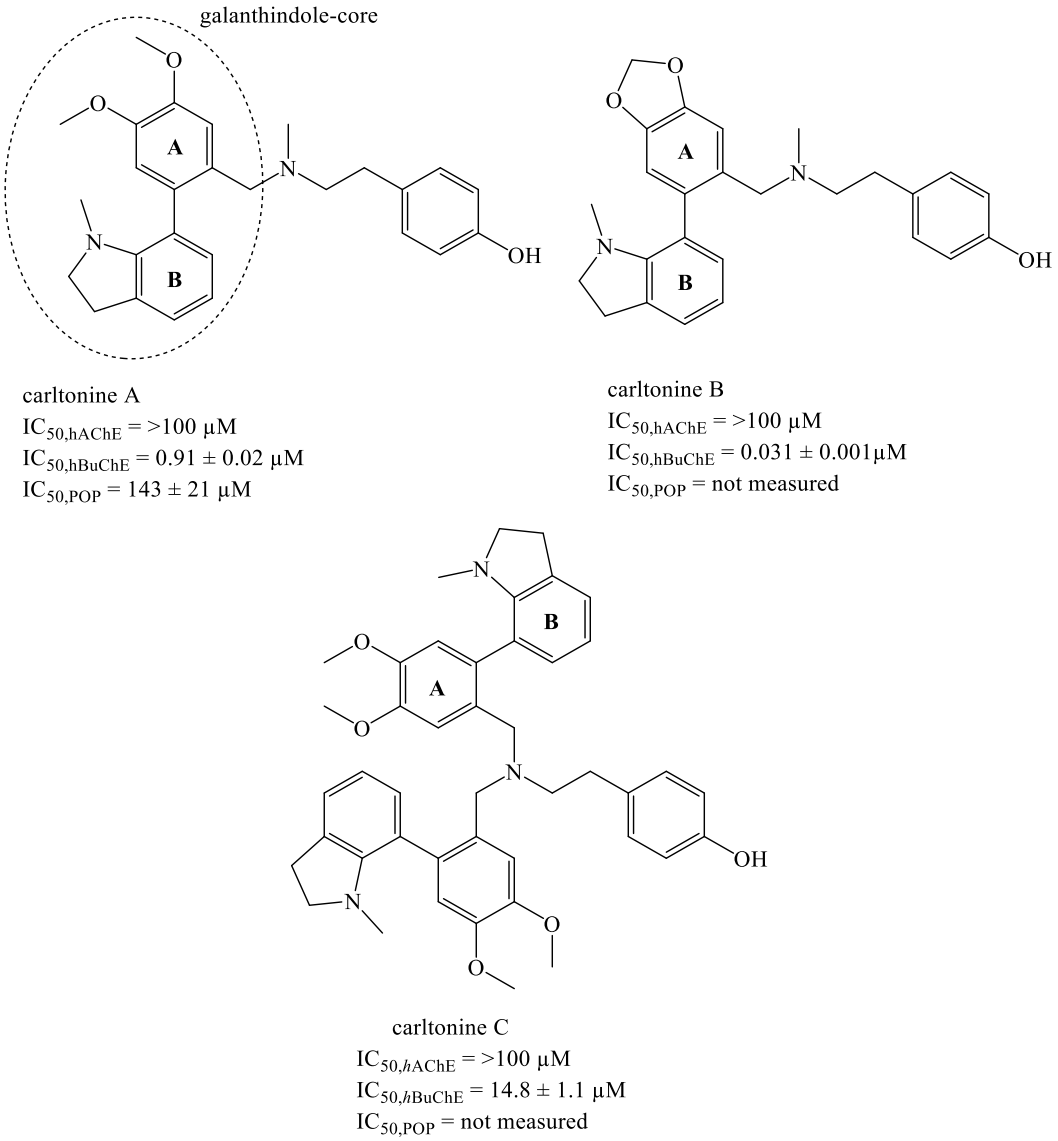


Fig. 5. Structures of carltonine-type AAs along with their biological activities connected with AD.

3.3 Genus *Hippeastrum* Herb. (Amaryllidaceae)

3.3.1 Geographical distribution, taxonomical aspects, and ethnobotany

The plants of the *Hippeastrum* genus are distributed from Mexico and the West Indies to Argentina, the majority in eastern Brazil, the Peruvian Andes, and Bolivia [63; 64]. It consists of large herbs of annual leaves, mostly hysteranthous, sessile, rarely persistent, and subpetiolate. Generally, the leaves are more than 2 cm wide. The scape is hollow with 2 free bracts. The flowers (2-13) are usually large and mostly purple or red. They are funnelform, zygomorphic, declinate, usually with a short tube and paraperigonal fibriae or with a callose ridge present at the throat. The stamens are fasciculate and declinate-ascendent. The stigma is trifid or shortly 3-lobed. The seeds are dry, flattened, obliquely winged, or irregularly discoid, hardly ever turgid and globose or subglobose, with a brown or black phytomelanous testa [63; 64]. A diploidism of 2n=22 is characteristic of the *Hippeastrum* genus, which is inarguably monophyletic except for a single species, *H. blumenavium*. This was first described as *Griffinia blumenavia* and further studies are required to clarify its correct position [65]. The beauty of their flowers has led to numerous *Hippeastrum* species being grown as ornamentals after hybridization although in horticultural circles the use of the name "*Amaryllis*" for this genus persists [64; 66].

The genus *Hippeastrum* Herb. is an ornamental Amaryllidaceae genus, which comprises approximately 60 species, about 30 of which are found in Brazil [67]. Plants of the genera *Hippeastrum* commonly called amaryllis, and *Amaryllis belladonna*, commonly called belladonna lily or naked lady, are similar in appearance except that *Amaryllis belladonna* has a solid flower stem while genus *Hippeastrum* has a hollow one [68]. Plants are known as “amaryllis“, “Dutch amaryllis“, and “giant amaryllis“ belong to the genus *Hippeastrum*, and those grown today are mostly hybrids of several species from South America and South Africa. There are many named varieties of *Hippeastrum* hybrids offering an array of flower colors and flower forms [68]. Most commercial cultivars are complex hybrids, with cultivars of *Hippeastrum* x *hybridum*, whose original species *H. vittatum* from Peru, suffered under hybridization with other species [69]. Plants of this genus have been traditionally used to cure tumors, hemorrhoids, and various inflammatory disorders such as asthma [70]. In northeastern Argentina, some species of the Amaryllidaceae family are used in traditional medicine by the Toba indigenous community, including the bulbs of *H. parodii* Hunz. & Cocucci for skin disorders (pimples, warts, and skin spots) [71].

The antitumor properties of the Amaryllidaceae alkaloids, such as lycorine, haemanthamine, and pancratistatine, are well known [72]. Lycorine and haemanthamine are easily isolated from natural sources and displayed significant *in vitro* cytotoxic activity against several

different types of cancer cell lines including MOLT-4, Hep-G2, HeLa, MCF-7, CEM, K562, A549, Caco-2, and HT-29 [11; 73]. Montanine, which has been isolated also from the genus *Hippeastrum*, showed promising cytotoxic activity on a panel of different cancerous cell lines [17]. Secondary metabolites from *H. puniceum* may also have therapeutic properties as it has been used in folk medicine to treat swellings and wounds [74].

3.3.2 Selected species of *Hippeastrum* plants

In the following section is summarized a short botanical description of phytochemically investigated *Hippeastrum* species.

3.3.2.1 *Hippeastrum argentinum* (Pax) Hunz.

The species *H. argentinum* is native to the Andean mountain environment found in Argentina and Bolivia. The flowers appear in the spring. At the top of the stem growing to a maximum height of 90 cm are white flowers in the number of 4-6. The flowers are funnel-shaped and reach a size of 20 cm [75; 76].

3.3.2.2 *Hippeastrum aulicum* (Ker Gawl.) Herb.

The species *H. aulicum* is native from Brazil south to Paraguay. It is an epiphytic species and grows on rocks and large trees in its native environment [77]. Sometimes it can be found on the ground. The bulbs have an ovoid shape with a short neck and measure up to 7.5 cm in diameter. The leaves are bright green, about 45 cm long, and 3.8-5 cm broad. The segments are bright crimson, green at the base; outer segments and lowest inner about an inch broad above the middle; 2 upper inner much broader. Stamens shorter than the segments; filaments bright red; anthers 1.3-1.9 cm (0.5-0.75 in.) long [76; 77].

3.3.2.3 *Hippeastrum breviflorum* Herb.

The species *H. breviflorum* is naturally occurring in the northwestern state of Argentina in South America [78]. The flowers are white, green toned, with a wider red stripe in the middle of each petal. The red stripe is further divided into two parts by a narrower white stripe. The flowers are placed on a frosted stem up to 90 cm long. The head with the scar on its top is shorter than the inflorescence, the stamens are even shorter compared to the head [78].

3.3.2.4 *Hippeastrum glaucescens* (Mart. ex Schult. & Schult. f.) Herb.

The species *H. glaucescens* is native to Brazil, flowering in spring. It grows only to a height of 40-60 cm. It has zygomorphic flowers. The inflorescence consists of 4 different sizes of petals colored green at the base and brick red at the edges. The species got its name thanks to the typically occurring green frosted leaves (glaucous - frosted) [75; 76].

3.3.2.5 *Hippeastrum morelianum* Lem.

The species *H. morelianum* is native to São Paulo, Brazil. A relatively long flowering period is typical, usually from spring to autumn. The stem reaches a length of 50 cm. At the top of the stem are 2 flowers up to 15 cm in size. The flowers are orange-red to red, from the base towards the tops of the petals is the green central area [79].

3.3.2.6 *Hippeastrum papilio* (Ravenna) Van Scheepen

The species *H. papilio* is native to tropical forests on the Atlantic coast in southern Brazil. It blooms in the late winter months until early spring [76]. This species is a robust, richly leafed species. At the top of the 50 cm, the high stem is 2-3 distinctly colored flowers with an average size of 12 cm. The color of the flowers can vary from white, through creamy green, to dark green with crimson, maroon, or purple stripes. The shape of the petals resembles the shape of a butterfly. The high growth rate of seedlings makes them an important species for the production of new hybrids. However, some crossing with existing hybrids leads to the production of seeds, which first develop intensively, but then growth is interrupted due to chromosomal incompatibilities [76; 80].

3.3.2.7 *Hippeastrum solandriflorum* (Lindl.) Herb.

The species *H. solandriflorum* grows in the north of South America, south to Peru. It blooms mainly in winter, but sometimes also in the summer months [77]. The onions are ovoid with a short neck and measure up to 7-10 cm in diameter. The leaves are elongated, grow to a length of 50 cm, width 2–2.5 cm. The stem reaches a length of 40-60 cm, surrounded by 2-6 flowers. The horizontal white flowers in the inflorescence have a funnel-shaped shape and are 18–25 cm in size. The stamens are shorter than the inflorescence, the stem reaches the length of the inflorescence [77].

3.3.2.8 *Hippeastrum vittatum* (L'Hér.) Herb.

The species *H. vittatum* is typically growing in South America's Peru and Brazil. It is an ornamental plant flowering in winter [81]. The onions are more or less regularly spherical, measuring between 5-8 cm in diameter. Elongated, light green, sessile leaves measure 45–60 cm in length and are 2.5–3.5 cm wide. The stem is straight and 60 to 100 cm long, bearing 2-6 flowers around 9-15 cm large flowers are the main decoration of the plant. They are white or pink, red striped, and have a funnel shape. The flower occupies a declining position in the inflorescence. The stamens are shorter than the petals, the head, together with the scar at its apex, reaches the length of the petals [75; 77].

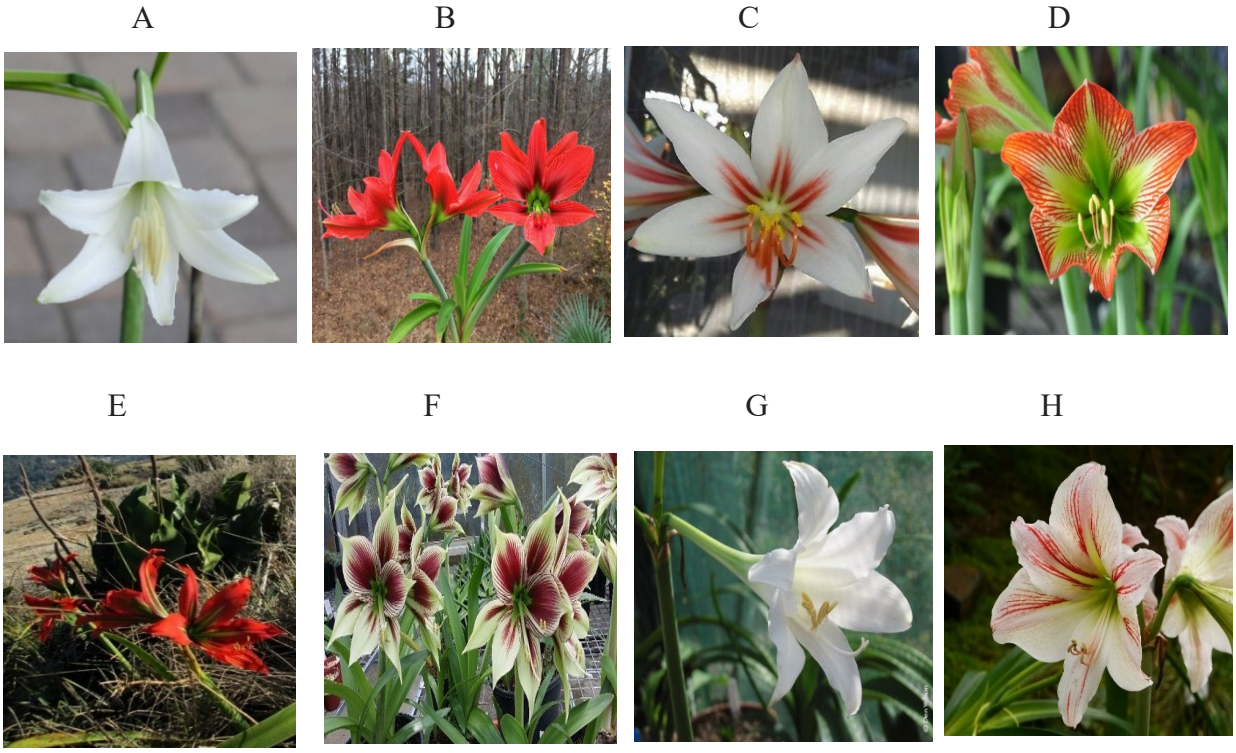


Fig. 6. Selected species from the genus *Hippeastrum* A (*Hippeastrum argentinum)* [76], B *(Hippeastrum aulicum)* [82], C *(Hippeastrum breviflorum)* [83], D (*Hippeastrum glaucescens*) [84], E (*Hippeastrum morelianum)* [85], F (*Hippeastrum papilio*) [80], G (*Hippeastrum solandriflorum*)[76], H (*Hippeastrum vittatum)* [86] .

Further *Hippeastrum* species that have been at least minimally studied from a phytochemical point of view include: *H. bifidum* (Herb.) Baker, *H. puniceum* (Lam.) Voss, *H. psittacinum* (Ker Gawl.) Herb, *H. striatum* (Lam.) H.E.Moore, *H. reticulatum* (L'Hér.) Herb., *H. calyptratum* (Ker Gawl.) Herb., *H. santacatarina* (Traub) Dutilh, *H. bicolor* (Ruiz & Pav.) Baker, *H. ananuca* Phil., *H. johnsonii*, *H. candidum* Stapt, *H. brachyandrum* Baker, *H. rutilum* (Ker Gawl.) Herb.

3.3.3 Phytochemistry of the genus *Hippeastrum*

Phytochemical studies of the genus *Hippeastrum*, as well as of other genera of the Amaryllidaceae family, started in the early 1950s. To the best of our knowledge, from approximately 60 *Hippeastrum* species described, only 29 including cultivated varieties have been phytochemically studied to date. Particular attention has been given to the study of alkaloids, and only little to other constituents. Bulbs from many species of the genus *Hippeastrum* yielded numerous alkaloids, with 64 being fully characterized. Further alkaloids have been identified using different spectroscopic methods including GC/MS, HPLC/MS. The described alkaloids belong to lycorine (**1-11**), homolycorine (**12-27**), haemanthamine (**28-37**), montanine (**38-40**), crinine (**41-43**), tazettine (**44-49**), narciclasine (**50-51**), galanthamine (**52-63**) and miscellaneous structural types (**64-69**). The following table (Table 2) and figures (Fig. 7-10) are a summary of alkaloids isolated from most studies species of the genus *Hippeastrum*.

The most studied species is *H. vittatum*, the first phytochemical study was published in 1954. This pilot study yielded in the isolation of the alkaloids lycorine (**1**) and tazettine (**44**) [87]. Two years later, the phytochemical study of the same species gave alkaloids haemanthamine (**28**), homolycorine (**12**), hippeastrine (**13**), and vittatine (**29**), as well as lycorine (**1**) and tazettine (**44**) [88]. Further study was carried out with *H. vittatum* growing in Egypt which allowed the elucidation of the alkaloids pancracine (**38**) (formerly hippagine) and hippadine (**6**) [89-91]. Phytochemical studies of *H. vittatum* flowers in 2001 yielded a representative alkaloid of ismine (**64**), the new alkaloid *O*-methylismine (**65**), and alkaloid of the carboline grouped named vittacarboline (**66**) [92]. The known compounds were isolated in 2011, namely 8-*O*-demethylmaritidine (**30**), 11-hydroxyvittatine (**31**), montanine (**39**), trisphaeridine (**50**), galanthamine (**52**), and sanguinine (**56**) [93].

Table 2. Alkaloids were reported in the selected species of genus *Hippeastrum*.

	H. argentinum	*H. aulicum*	*H. breviflorum*	*H. glaucescens*	*H. morelianum*	*H. papilio*	*H. solandriflorum*	*H. vittatum*
Lycorine-type								
Lycorine (**1**)	[94]	[95-97]		[98]			[99]	[87-89; 93; 100; 101]
Pseudolycorine (**2**)		[95; 96]					[102]	[93]
Galanthine (**3**)		[95; 97]						
Norpluviine (**4**)		[95; 97]						
11,12-Dehydroanhydrolycorine (**5**)	[94]		[101]					[93]
Hippadine (**6**)								[89; 90]
Hippacine (**7**)								[89; 103; 104]
Narcissidine (**8**)		[95]					[102]	
Anhydrolycorine (**9**)	[94]	[96]						[101]
Ungeremine (**10**)							[99]	
Incartine (**11**)		[97]						
Homolycorine-type								
Homolycorine (**12**)		[95-97]						[88]
Hippeastrine (**13**)				[101]				[88; 89]
Candimine (**14**)					[101; 105]			
2*α*-10b*α*-Dihydroxy-9-*O*-demethylhomolycorine (**15**)							[102]	
8-*O*-Demethylhomolycorine (**16**)		[97]	[101]					
Nerinine (**17**)		[96; 97]			[101]			
2-Hydroxyhomolycorine (**18**)					[101]			
2*α*-Methoxyhomolycorine (**19**)		[97]			[101]			
2*α*,7-Dimethoxyhomolycorine (**20**)					[101; 105]			
Deoxylycorenine (**21**)			[101]					
Hippapiline (**22**)						[106]		
Papiline (**23**)						[106]		

Table 2. *Cont.*	***H. cargentinum***	***H. aulicum***	***H. breviflorum***	***H. glaucescens***	***H. morelianum***	***H. papilio***	***H. solandriflorum***	***H. vittatum***
7-Methoxy-*O*-methyllycorenine (**24**)		[96; 97]						
Albomaculine (**25**)		[96; 97]						
7-Hydoxyclivonine (**26**)	[94]							
O-Methyllycorenine (**27**)		[97]						
Haemanthamine-type								
Haemanthamine (**28**)		[96; 97]			[105]	[101; 106; 107]		[88]
Vittatine (**29**)	[94]	[96; 97]	[101]			[101; 107]	[99]	[88; 89; 93; 100; 101; 104]
8-*O*-Demethylmaritidine (**30**)		[96; 97]				[101; 107]		[93; 101]
11-Hydroxyvittatine (**31**)		[97]				[101; 107]	[102]	[93]
Hamayne (**32**)	[94]	[97]			[105]		[99]	
3-*O*-Demethyl-3-*O*-(3-hydroxybutanoyl)haemanthamine (**33**)						[106]		
11-Oxohaemanthamine (**34**)		[96; 97]						
Crinamine (**35**)		[97]						
Haemanthidine (**36**)		[97]						
6-Epihaemanthidine (**37**)		[97]						
Montanine-type								
Pancracine (**38**)	[94]							[89; 91; 93; 101]
Montanine (**39**)	[94]	[95]						[93; 100; 101]
4-*O*-Methylnangustine (**40**)	[94]							
Crinine-type								
Ambelline (**41**)		[95]						
Aulicine (**42**)		[96; 97]						
Buphanisine (**43**)		[97]						
Tazettine-type								
Tazettine (**44**)		[96; 97]	[101]	[98; 101]	[101; 105]	[101]		[87-89]
Pretazettine (**45**)		[96; 97]		[98]	[105]			

Table 2. *Cont.*

	H. argentinum	*H. aulicum*	*H. breviflorum*	*H. glaucescens*	*H. morelianum*	*H. papilio*	*H. solandriflorum*	*H. vittatum*
3-*Epi*-macronine (**46**)		[97]	[101]	[101]	[101; 105]			
3-*Epi*-deoxytazettine (**47**)			[101]	[101]	[101]			
Deoxytazettine (**48**)			[101]	[101]	[101]			
6-Methoxypretazettine (**49**)			[101]		[101]			
Narciclasine-type								
Trisphaeridine (**50**)		[96; 97]	[101]	[101]	[101; 105]	[101]		[93]
Narciclasine (**51**)							[102]	
Galanthamine-type								
Galanthamine (**52**)	[94]	[96; 97]		[101]	[101]	[101; 106; 107]	[102]	[93; 101]
Apogalanthamine (**53**)						[106]		
Chlidanthine (**54**)		[95]						
N-Formylnorgalanthamine (**55**)				[101]				
Sanguinine (**56**)				[101]		[101]	[102]	[93]
N-Demethylgalanthamine (**57**)				[101]				
3-*Epi*-norgalanthamine (**58**)				[101]				
Narwedine (**59**)		[96; 97]		[101]		[101; 106; 107]		
11β-Hydroxygalanthamine (**60**)						[101; 106; 107]		
Anhydrogalanthamine (**61**)				[101]				
Lycoramine (**62**)		[97]						
Galanthamine *N*-oxide (**63**)							[102]	
Miscellaneous								
Ismine (**64**)		[96; 97]	[101]		[101]		[99]	[92; 93]
O-Methylismine (**65**)								[92]
Vittacarboline (**66**)								[92]
Galanthindole (**67**)			[101]		[101]			
Lycosinine B (**68**)			[101]					
9-*O*-Demethyllycosinine B (**69**)						[106]		

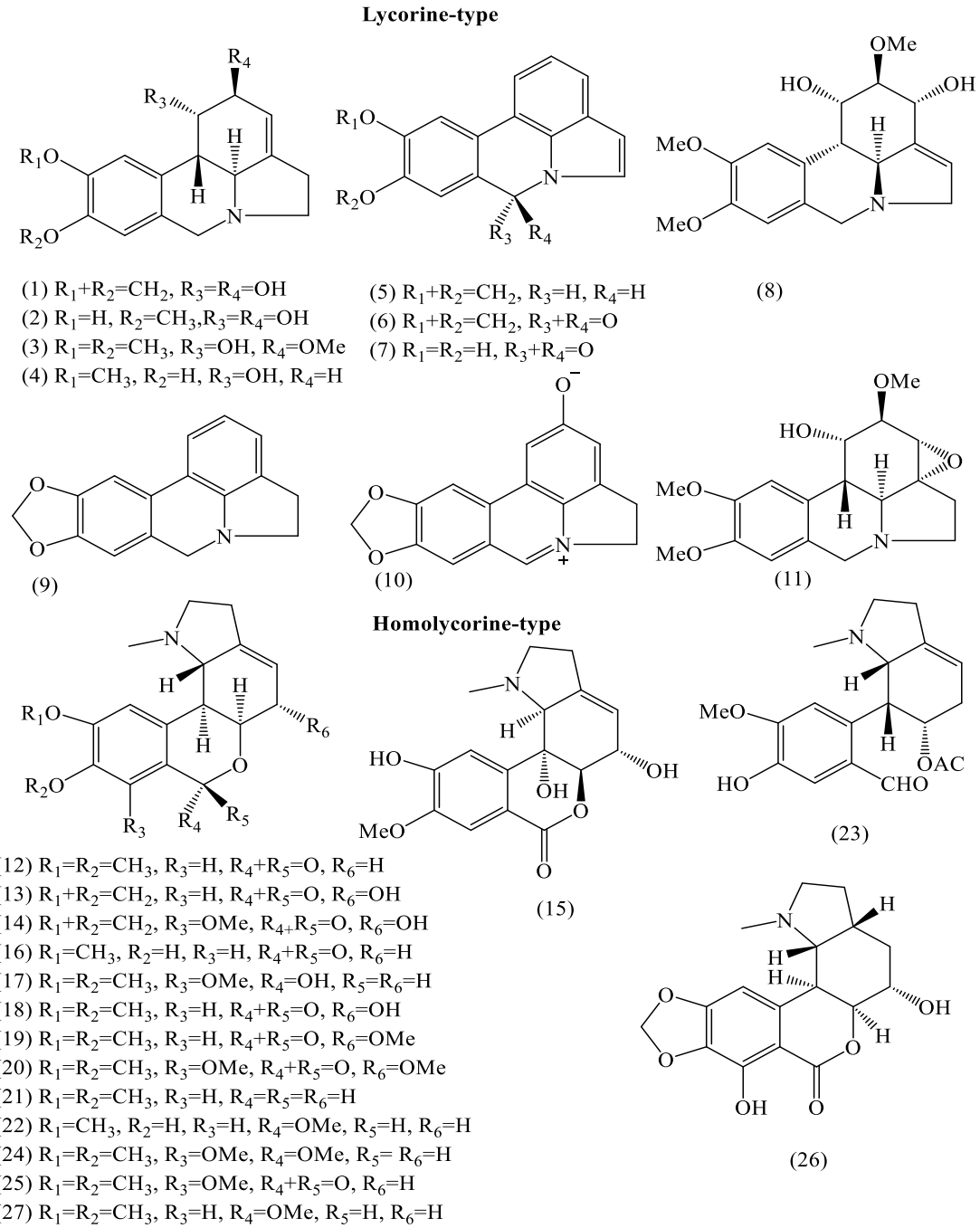


Fig. 7. Amaryllidaceae alkaloids of lycorine- and homolycorine-type were reported in the selected species of genus *Hippeastrum*.

Various phytochemical studies carried out on *H. aulicum* revealed the presence of several types of AAs, including lycorine (**1**), pseudolycorine (**2**), galanthine (**3**), norpluviine (**4**), narcissidine (**8**), anhydrolycorine (**9**), incartine (**11**), homolycorine (**12**), 8-*O*-demethylhomolycorine (**16**), nerinine (**17**), 2*α*-methoxyhomolycorine (**19**), 7-methoxy-*O*-methyllycorenine (**24**), albomaculine (**25**), *O*-methyllycorenine (**27**), haemanthamine (**28**), vittatine (**29**), 8-*O*-methylmaritidine (**30**), 11-hydroxyvittatine (**31**), hamayne (**32**), 11-oxohaemanthamine (**34**), crinamine (**35**), haemanthidine (**36**), 6-epihaemanthidine (**37**),

montanine (**39**), ambelline (**41**), aulicine (**42**), buphanisine (**43**), tazettine (**44**), pretazettine (**45**), 3-*epi*-macronine (**46**), trisphaeridine (**50**), galanthamine (**52**), chlidanthine (**54**), narwedine (**59**), lycoramine (**62**), and ismine (**64**) [95-97].

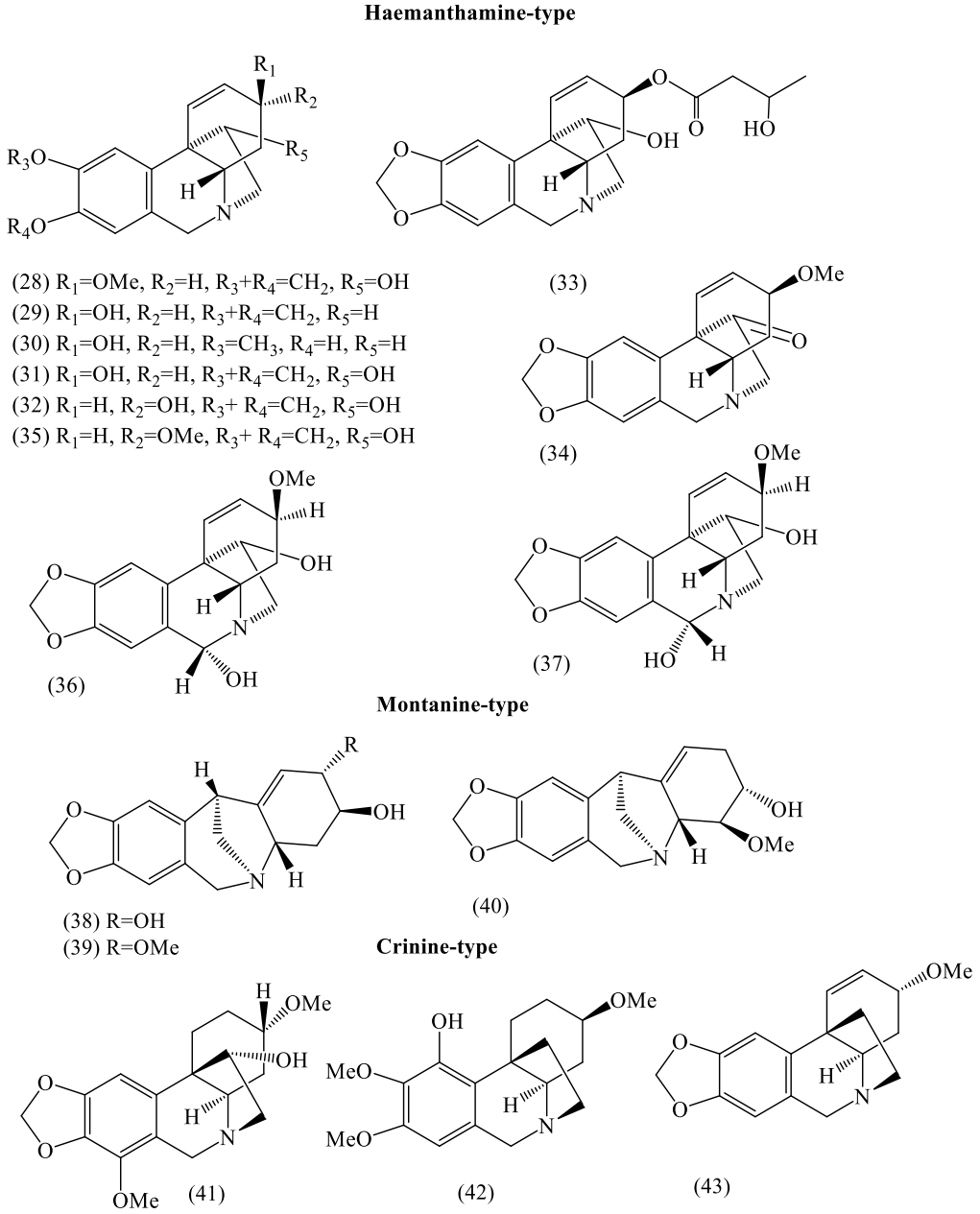


Fig. 8. Amaryllidaceae alkaloids of haemanthamine-, montanine- and crinine-type were reported in the selected species of genus *Hippeastrum*.

Several types of AAs have been isolated from *H. solandriflorum* including a new one alkaloid 2*α*-10b*α*-dihydroxy-9-*O*-demethylhomolycorine (**15**). In addition to this new alkaloid twelve known AAs of different structural types were isolated: lycorine (**1**), pseudolycorine (**2**), narcissidine (**8**), ungeremine (**10**), vittatine (**29**), 11-hydroxyvittatine (**31**), hamayne (**32**),

narciclasine (**51**), galanthamine (**52**), sanguinine (**56**), galanthamine *N*-oxide (**63**) and ismine (**64**) [99; 102].

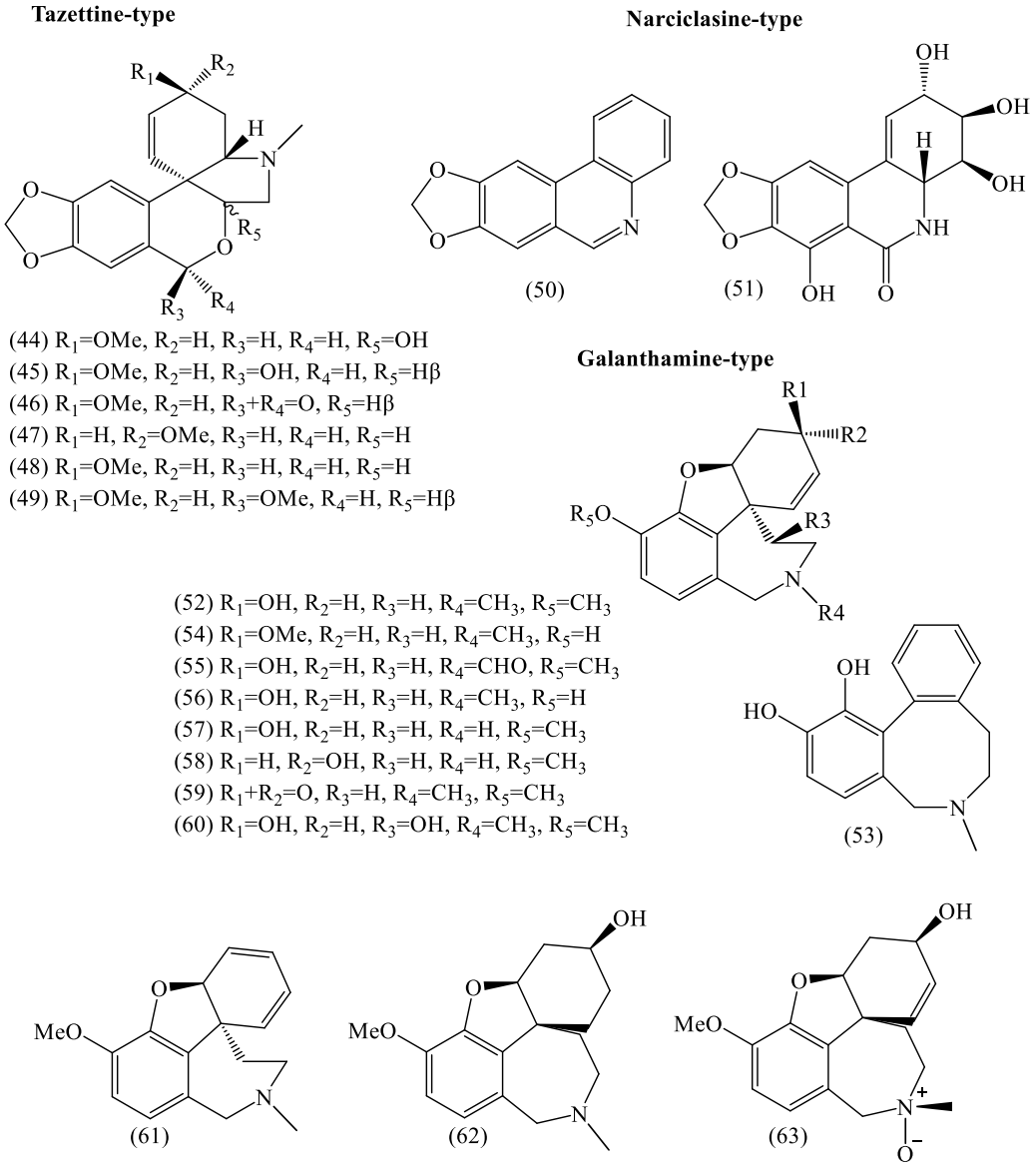


Fig. 9. Amaryllidaceae alkaloids of, tazettine-, narciclasine- and galanthamine-type were reported in the selected species of genus *Hippeastrum*.

Several alkaloids were isolated from *H. glaucescens*, namely lycorine (**1**), hippeastrine (**13**), tazettine (**44**), pretazettine (**45**), 3-*epi*-macronine (**46**), 3-*epi*-deoxytazettine (**47**), deoxytazettine (**48**), trisphaeridine (**50**), galanthamine (**52**), *N*-formylnorgalanthamine (**55**), sanguinine (**56**), *N*-demethylgalanthamine (**57**), 3-*epi*-norgalanthamine (**58**), narwedine (**59**) and anhydrogalanthamine (**61**) [98; 101].

Phytochemical investigation of *H. morelianum* plant revealed the presence of seventeen alkaloids including candimine (**41**), nerinine (**17**), 2-hydroxyhomolycorine (**18**), 2*α*-methoxyhomolycorine (**19**), 2*α*, 7-dimethoxyhomolycorine (**20**), haemanthamine (**28**), hamayne

(**32**), tazettine (**44**), pretazettine (**45**), 3-*epi*-macronine (**46**), 3-*epi*-deoxytazettine (**47**), deoxytazettine (**48**), 6-methoxypretazettine (**49**), trisphaeridine (**50**), galanthamine (**52**), ismine (**64**) and galanthindole (**67**) [101; 105].

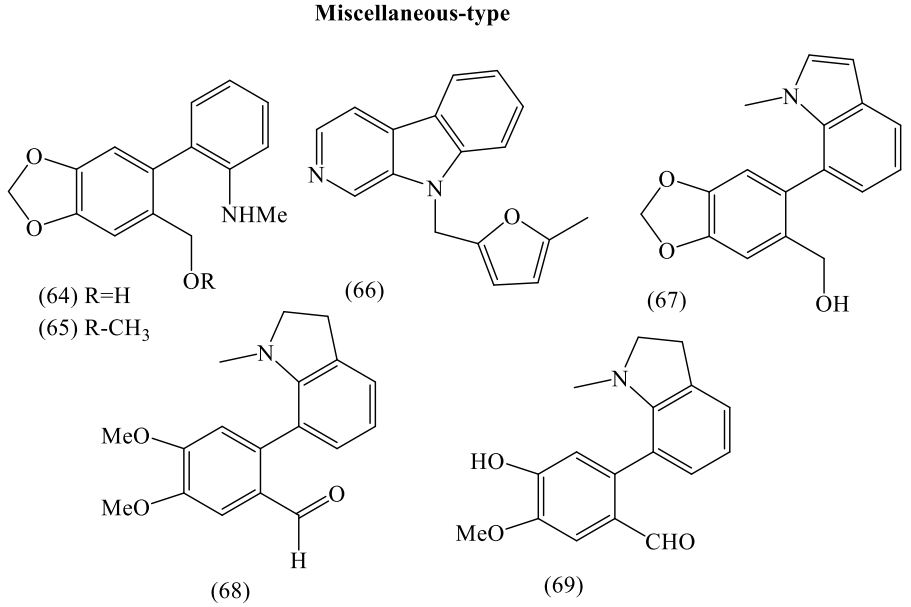


Fig.10. Amaryllidaceae alkaloids of other structural types reported in the selected species of genus *Hippeastrum*.

Phytochemical investigation of *H. papilio* bulbs leading to the identification of hippapiline (**22**), papiline (**23**), and 3-*O*-demethyl-3-*O*-(3-hydroxybutanoyl)haemanthamine (**33**) as the novel constituents. In addition to twelve other known AAs, haemanthamine (**28**), vittatine (**29**), 8-*O*-demethylmaritidine (**30**), 11-hydroxyvittatine (**31**), tazettine (**44**), trisphaeridine (**50**), galanthamine (**52**), Apogalanthamine (**53**), sanguinine (**56**), narwedine (**59**), 11β-hydroxygalanthamine (**60**) and 9-*O*-demethyllycosinine B (**69**) [101; 106; 107]. Several types of alkaloids were isolated from *H. breviflorum* including 11,12-dehydroanhydrolycorine (**5**), 8-*O*-demethylhomolycorine (**16**), deoxylycorenine (**21**), vittatine (**29**), tazettine (**44**), 3-*epi*-macronine (**46**), 3-*epi*-deoxytazettine (**47**), deoxytazettine (**48**), 6-methoxypretazettine (**49**), trisphaeridine (**50**), ismine (**64**), galanthindole (**67**) and lycosinine B (**68**) [101].

Several types of alkaloids were isolated and identified from *H. argentinum* plant, two new alkaloids, 7-hydroxyclivonine (**26**), and 4-methylnangustine (**40**). In addition to the known compounds, including lycorine (**1**), vittatine (**29**), hamayne (**32**), pancracine (**38**), montanine (**39**), 11,12-dehydroanhydrolycorine (**5**), anhydrolycorine (**9**), and galanthamine (**52**) [94].

Based on GC/MS screening studies performed on different commercially available *Hippeastrum* cultivars [17], *Hippeastrum* x *hybridum* cv. Ferrari has been selected for detailed phytochemical study as a source of the wide range of biologically active AAs. The GC/MS

analysis of this cultivar demonstrated a high concentration of haemanthamine-type alkaloids such as vittatine and 11-hydroxyvittatine [17]. In our previous research, the simple semisynthetic derivatives of haemanthamine displayed promising inhibitory activities against cholinesterases [108]. Thus the next step of the study was the preparation of further derivatives of the haemanthamine-type AAs, vittatine, on a pilot scale, to develop new lead-structures for the potential treatment of AD, since the portfolio of used drugs for AD is narrow.

3.3.4 Biological activities of alkaloids isolated from *Hippeastrum* plants

As mentioned before, AAs have shown a wide range of bioactivity, including antiviral, cytotoxic, antitumoral, and pro-apoptotic, as well as antimalarial, anti-inflammatory, antimicrobial, and AChE inhibiting activities [109]. Thus, different extracts and AAs isolated from various *Hippeastrum* species were screened for various biological activities within several studies.

3.3.4.1 Biological activity connected with Alzheimer´s disease

Cholinesterase inhibitors are the only approved drugs for treating patients with mild to moderately severe Alzheimer's disease, a disorder associated with progressive degeneration of memory and cognitive function. The cholinergic hypothesis postulates that memory impairment in patients with Alzheimer's disease results from a deficit of cholinergic function in the brain [110; 111]. The most important changes observed in the brain are a decrease in hippocampal and cortical levels of the neurotransmitter acetylcholine and associated enzyme choline transferase. Acetylcholinesterase inhibitors can restore the level of acetylcholine by inhibiting acetylcholinesterase.

One of the most important substances with the ability to inhibit AChE is Amaryllidaceae alkaloid galanthamine (**52**), originally isolated from the bulbs of *Galanthus woronowii* Losinsk., and later from other plants of the family [49].

Given the therapeutic success of galanthamine (**52**), plants of the Amaryllidaceae family invoke widespread interest as an interesting source for further AChE/BuChE inhibitors.

The ethanol extract and different alkaloid-rich fractions prepared from bulbs of *H. psittacinum* were evaluated for their AChE inhibition. Ethanol extract demonstrated AChE inhibitory activity with the value of IC_{50} 11.2 ± 0.7 μg/mL. The GC-MS analysis of this extract showed the presence of AAs of different structural types. As major components of the extract were identified homolycorine-type alkaloid albomaculine (**25**) and narciclasine-type trisphaeridine (**50**). The mentioned extract has been also tested for its neuroprotective and

genotoxic activities in SH-SY5Y cells to determine if the extract protected cells from oxidative damage caused by H_2O_2 [112].

Pretazettine (**45**) and hippeastrine (**13**) isolated from fresh bulbs of *H. psittacinum*, together with montanine (**39**) isolated from *H. vittatum*, were screened for their AChE inhibition potency using AChE isolated from rats brains. Montanine (**39**) inhibited, in a dose dependent manner, more than 50 % of the enzyme at 1mM concentration, while hippeastrine (**13**) and pretazettine (**45**) showed no significant AChE inhibition [113; 114].

Seven alkaloids isolated from fresh bulbs of *H. papilio* including 11-hydroxygalanthamine (**60**), galanthamine (**52**), narwedine (**59**), haemanthamine (**28**), 11-hydroxyvittatine (**31**), 8-*O*-demethylmaritidine (**30**), and vittatine (**29**) were screened for their AChE inhibition activity using modified Ellman´s method and commercially available AChE from an electric eel. Within tested alkaloids, only 11-hydroxygalanthamine (**60**) showed promising AChE inhibition potency with the value of IC_{50} = 14.5 ± 0.33 μM, while galanthamine (**52**) showed an IC_{50} of 1.18 ± 0.07 μM [107].

Seven alkaloids isolated from the bulbs of *H. reticulatum*, among them *N*-chloromethylnarcissidinium (**70**), narciprimin (**71**), *N*-methyltyramine (**72**), and 3*β*,11*α*-dihydroxy-1,2-dehydrocrinane (**73**) showed AChE inhibition with IC_{50} values of 29.1, 46.4, 70.1 and 104.5 μg/mL, respectively. The effect of the alkaloid fraction of *H. reticulatum* (AHR) on cognitive and memory deficits in a scopolamine-induced rodent has been also studied [115] (Fig.11).

Within another study from 2015, six AAs have been isolated from bulbs of *H. argentinum*, and tested for their acetyl- and butyrylcholinesterase inhibitory potency. Isolated alkaloids including 4-*O*-methylnangustine (**40**), 7-hydroxyclivonine (**26**), pancracine (**38**), montanine (**39**), hamayne (**32**), and lycorine (**1**). Within tested alkaloids, only 7-Hydroxyclivonine (**26**) showed weak butyrylcholinesterase inhibitory activity, with a value of IC_{50} = 67.3 ± 0.09 μM, whereas remaining alkaloids were considered as inactive against both enzymes [94].

3.3.4.2 Cytotoxic activity

Oncological diseases are one of the leading causes of death in the developed countries and the increase of its prevalence seems to be inevitable. In most cases, oncological patients die due to the resistance of cancer to therapy, metastasis, and dissemination of cancer cells into vital organs. The standard treatment covers surgical intervention, radiotherapy, and/or chemotherapy. Additionally, conventional anticancer treatments damage healthy tissue, resulting in a variety of

side effects. Therefore, substantial efforts are being invested into identifying and developing compounds that would be able to selectively target tumor cells while not damage healthy cells.

The dichloromethane (CH_2Cl_2) and *n*-butanol extracts obtained from fresh bulbs of *H. vittatum*, were screened for their cytotoxic activity in *vitro*, against five human cell lines (HT29 colon adenocarcinoma; H460 non-small cell lung carcinoma; RXF393 renal cell carcinoma; MCF7 breast cancer; and OVCAR3 epithelial ovarian cancer), using the sulphorhodamine B assay. Both extracts showed potential antiproliferative activity. From CH_2Cl_2 fraction, two alkaloids were isolated including vittatine (**29**), and montanine (**39**). The alkaloids were submitted to the antiproliferative assay and the highest level of cytotoxicity was found for the alkaloid montanine (**39**) [100] (Table 3).

Table 3. Cytotoxic activity of CH_2Cl_2 and *n*-butanol extracts and isolated alkaloids of *Hippeastrum vittatum* [100].

Sample	**Cell lines[a]**				
	HT29	**H460**	**RXF393**	**MCF7**	**OVCAR3**
CH_2Cl_2	0.68 ± 0.21	0.62 ± 0.06	0.79 ± 0.52	1.60 ± 0.34	0.84 ± 0.29
n-Butanol	4.08 ± 0.61	3.34 ± 0.30	2.93 ± 0.6	3.74 ± 0.29	3.56 ± 0.25
Montanine (**39**)	0.71 ± 0.10	0.57 ± 0.57	0.65 ± 0.01	0.74 ± 0.02	0.84 ± 0.11
Vittatine (**29**)	21.91 ± 1.61	15.88 ± 3.28	29.57 ± 12.66	NT	NT

[a]Results are expressed as IC_{50} values (µg/ml), means of 3 determinations, measured by SRB; activity: < 5 strong, 5–20 moderate, 20–50 weak, > 50 inactive. Key to cell lines employed: HT29 (colon adenocarcinoma), H460 (non-small cell lung carcinoma), RXF393 (renal cell carcinoma), MCF7 (breast cancer), and OVCAR3 (epithelial ovarian cancer). NT: not tested.

Four AAs isolated from bulbs of *H. goianum*, namely 7-deoxi-*trans*-dihydronarciclasine (**74**), 9-*O*-demethyllycoramine (**75**) (Fig.11), lycorine (**1**), and pseudolycorine (**2**) were screened for their cytotoxic activity against colorectal carcinoma (HCT 116), breast carcinoma (MCF-7), and non-tumor human retinal pigment epithelium (RPE) cell lines. The IC_{50} of 7-deoxy-*trans*-dihydronarciclasine (**74**) against each cell line was equivalent to the positive control doxorubicine, indicating a considerable cytotoxic activity. Results are summarized in the following table (Table 4) (Fig.11).

Table 4. Cytotoxic activity is expressed as IC_{50} of isolated alkaloids of *Hippeastrum goianum* [116].

Alkaloid	**Cell line/ IC_{50} (µM)**		
	HC-116	**MCF-7**	**RPE**
7-Deoxi-*trans*-dihydronarciclasine (**74**)	0.15	0.2	0.3
9-*O*-Demethyllycoramine (**75**)	> 50	> 50	> 50
Lycorine (**1**)	> 50	> 50	> 50
Pseudolycorine (**2**)	4.6	10.6	42.6
Doxorubicin	0.4	0.6	1.2

The chemical investigation of the ethanol extract from bulbs of *H. solandriflorum* allowed the isolation of eight alkaloids 2*α*-10b*α*-dihydroxy-9-*O*-demethylhomolycorine (**15**), pseudolycorine (**2**), narcissidine (**8**), 11-hydroxyvittatine (**31**), narciclasine (**51**), galanthamine (**52**), sanguinine (**56**), and galanthamine *N*-oxide (**63**). The overall cytotoxic effect of isolated alkaloids was screened using MMT assay against four cancer cell lines HCT-116 (colon adenocarcinoma), HL-60 (leukemia), OVCAR-8 (ovarian carcinoma), and SF-295 (glioblastoma). Among all of them, narciclasine (**51**) showed the highest cytotoxicity against the four cell lines tested, with IC_{50} values ranging from 0.01 to 0.09 µM. Besides narciclasine (**51**), only pseudolycorine (**2**) can be considered as highly cytotoxic, with IC_{50} values in the 1µM range. In general, HCT-116 was the most sensitive cell line, to which five of the eight tested compounds presented IC_{50} values below 50 µM [102] (Table 5).

Table 5. Cytotoxicity of the compounds isolated from *H. solandriflorum* on select tumor cell lines evaluated by the MTT assay after 72 h of exposure.

Alkaloid	**IC_{50}/µM**			
	HCT-116	**HL-60**	**OVCAR8**	**SF-295**
2*α*-10b*α*-Dihydroxy-9-*O*-demethylhomolycorine (**15**)	11.69	> 50	15.11	16.31
Pseudolycorine (**2**)	0.85	1.10	1.59	1.81
Narcissidine (**8**)	> 50	> 50	> 50	> 50
11-Hydroxyvittatine (**31**)	35.71	> 50	> 50	> 50
Narciclasine (**51**)	0.02	0.01	0.09	0.02
Galanthamine (**52**)	> 50	> 50	> 50	> 50
Sanguinine (**56**)	29.00	32.08	> 50	> 50
Galanthamine *N*-oxide (**63**)	> 50	> 50	> 50	> 50
Doxorubicin	0.02	0.03	0.29	0.33

Recently, a new glycosylated derivative narciclasine-4-*O*-*β*-D-xylopyranoside (**76**) has been isolated from the whole plant of *H. puniceum*, and screened for its cytotoxicity against colon (HC 116) and breast (MCF-7) tumor cells. Unfortunately, this new compound showed no significant cytotoxic activity [117] (Fig.11).

3.3.4.3 Further biological activities

Within the course of searching for natural anti-diabetic alternatives from plants, the potential hypoglycaemic effects of crude mucilage of *H. vittatum* bulbs were examined at 150, and 250 mg/kg using the streptozotocin-induced diabetic rat model. Overall, as compared to the untreated diabetic rats, oral administration of the mucilage at 150 mg/kg significantly ameliorated the induced hyperglycemia by 45.37 % and 61.95 % after 2 and 4 h, respectively, whereas the

maximum hypoglycemic actions were observed at 250 mg/kg, causing 57.37 % and 65.74 % reduction in the elevated blood glucose levels after 2 and 3 h, respectively [118]. Montanine (**39**), which has been isolated from *H. vittatum* showed anxiolytic, antidepressant, and anticonvulsant-like effects in mice [114]. Lycorine (**1**), the most frequently occurring alkaloid in almost all *Hippeastrum* species has been approved for several biological activities including antiviral, anti-inflammatory, antifungal, and antiprotozoal activities [119-122]. Homolycorine (**12**), and 8-*O*-demethylhomolycorine (**16**) have a hypotensive effect on the arterial pressure of normotensive rats [47]. Vittatine (**29**) has antibacterial activity against the Gram-positive *Staphylococcus aureus* and the Gram-negative *Escherichia coli* [47]. Some alkaloids such as trisphaeridine (**50**), possess high antiretroviral activities, accompanied by low therapeutic indices [47].

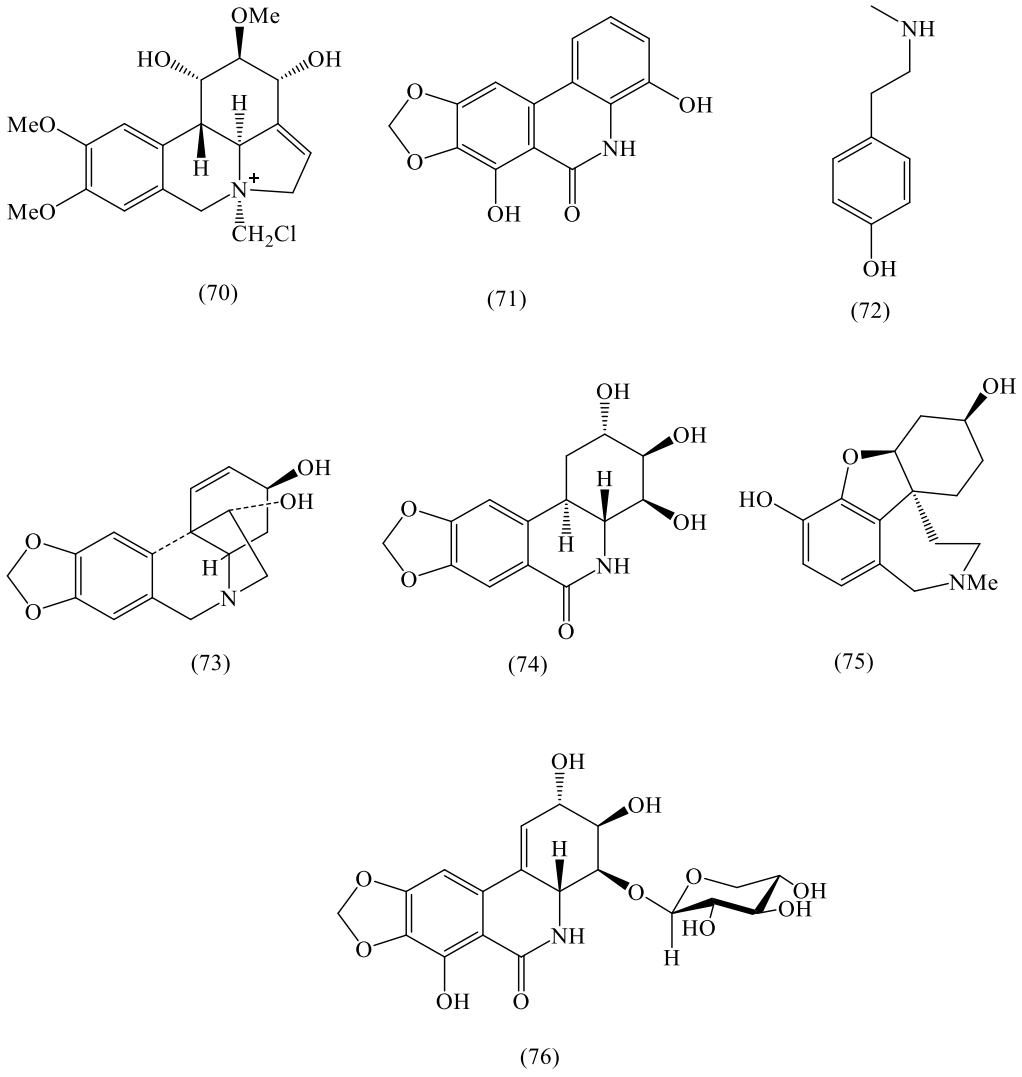


Fig.11. Further structures of AAs were reported in *H. reticulatum*, *H. goianum,* and *H. puniceum* respectively.

4 EXPERIMENTAL PART

4.1 Materials and instruments used for the isolation of Amaryllidaceae alkaloids

4.1.1 Chemicals used for the isolation of alkaloids

- Anhydrous sodium carbonate p. (Ing. Švec – Penta, Praha)
- Bismuthi subnitras (Lachema, Brno) ($BiNO_3(OH)_2$. $BiO(OH)$)
- Diethylamine f.a. (Ing. Švec – Penta, Praha) (Et_2OH, DEA)
- Hydrochloric acid 35% f.a. (Ing. Švec – Penta, Praha) (HCl)
- Mercuric chloride f.a. (Fisher Scientific, Pardubice) ($HgCl_2$)
- Potassium iodide p. (Lach-Ner, Neratovice) (KI)
- Sodium hydroxide p. (Ing. Švec – Penta, Praha) (NaOH)
- Sulfuric acid 96% f.a. (Ing. Švec – Penta, Praha) (H_2SO_4)
- L-(+)-Tartaric acid f.a. (Balex, Pardubice) ($C_4H_6O_6$)
- Water solution of ammonia 22-25% f.a. (Ing.Švec – Penta, Praha) (NH_4OH)

4.1.2 Solvents used for the isolation and structural identification of Amaryllidaceae alkaloids

- Acetone f.a. (Ing. Švec – Penta, Praha) (Me_2CO)
- Chloroform p. (Ing. Švec – Penta, Praha) ($CHCl_3$)
- Cyclohexane p. (Ing. Švec – Penta, Praha) (C_6H_{12}, cHx)
- Deuterated chloroform for NMR analysis (Sigma Aldrich, Praha) ($CDCl_3$)
- Deuterated methanol for NMR analysis (Sigma Aldrich, Praha) (CD_3OD)
- Diethyl ether f.a. (Ing. Švec – Penta, Praha) (Et_2O)
- Dioxane f.a. (Lach-Ner, Neratovice) ($C_4H_8O_2$)
- Ethanol 95% (Lihovar Chrudim, Chrudim) (EtOH)
- Ethyl acetate p. (Ing. Švec – Penta, Praha) (EtOAc)
- *n*-Hexane (Carlo Erba Reagents, Val-de-Reuil, France) (C_6H_{14})
- 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) ($C_6H_5CH_3$, To)
- Water ultrapure.

4.1.3 Adsorbents for column chromatography, analytical and preparative thin layer chromatography

- Aluminium oxide (Al_2O_3) neutral, for chromatography, 63 – 200 μm; Across (Lach-Ner, Neratovice). The activation of Al_2O_3 was performed at 200°C for 8 hours. After cooling, the active Al_2O_3 was deactivated by the addition of water (6 %) and mixed in a closed bowl for 1 hour.
- The silica gel: Silica gel 60 GF_{254} (Merck Millipore, Praha) was used for the preparation of the molten layer on the sheets.
- Commercial analytical TLC sheets Silica gel GF_{254} 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.

4.1.4 Mobile phases used for preparative and analytical TLC

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

Table 6. Mobile phases used for TLC

Mobile phase	Ingredients
MP-1	$CHCl_3$:MeOH:NH_3 (9.5:7.5:0.25)
MP-2	$CHCl_3$:MeOH:NH_3 (80:20:0.25)
MP-3	To:$CHCl_3$:DEA (85:10:5)
MP-4	To:EtOAc:DEA (90:5:5)
MP-5	$CHCl_3$:MeOH:NH_3 (85:15:0.25)
MP-6	$CHCl_3$:MeOH:NH_3 (75:25:0.25)
MP-7	To:EtOH:DEA (80:10:10)
MP-8	$CHCl_3$:MeOH:NH_3 (60:40:0.25)
MP-9	To:EtOAc:DEA (80:10:10)
MP-10	To:EtOAc:DEA (60:30:10)
MP-11	$CHCl_3$:MeOH:NH_3 (70:30:0.25)
MP-12	To:EtOAc:DEA (15:75:5)
MP-13	Chx:EtOAc:DEA (70:25:5)

4.1.5 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.

4.1.6 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)

4.1.7 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).

4.2 Materials and instruments used for the determination of biological activities

4.2.1 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_2O)
- Monopotassium phosphate anhydrous f.a. (Lach-Ner, Neratovice) (KH_2PO_4)
- Sodium dihydrogen phosphate f.a. (Ing. Švec – Penta, Praha) ($NaH_2PO_4 . 2H_2O$)
- Dimethyl sulfoxide f.a. (Sigma Aldrich, Germany) (DMSO)
- 5,5'-Dithiobis-(2-nitrobenzoic acid) ≥ 98% (Ellman's reagent) (Sigma Aldrich, Praha) (DTNB)
- Galanthamine hydrobromide > 98% (Changsha Organic Herb Inc., People Republic of China) ($C_{17}H_{21}NO_3$)
- Disodium phosphate anhydrous f.a. (Ing.Švec – Penta, Praha) (Na_2HPO_4)
- 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) ($C_{13}H_{16}N_4O_4$)
- *Z*-Pro-prolinal ≥ 98% (Sigma Aldrich, Praha) ($C_{18}H_{22}N_2O_4$)

4.2.2 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 NaH_2PO_4 (1.20 g of NaH_2PO_4 in 1 l of this solution). The stock solution B – 10 mM solution of Na_2HPO_4 (1.42 g of Na_2HPO_4 in 1 l of this solution).
- 5mM 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 NaH_2PO_4 (24.0 g of NaH_2PO_4 in 1 l of this solution). The stock solution B – 200 mM solution of Na_2HPO_4 (28.4 g of Na_2HPO_4 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_2PO_4 and 1.5 g sodium chloride and dissolved in 100 ml distilled water. Solution B was prepared by dissolving 0.89 g of NaH_2PO_4 2 H_2O 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.

4.2.3 Instruments used for 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, Tuttlingen, 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).

4.3 Plant material

The fresh bulbs of all *Hippeastrum* taxa (between 150 g – 250 g) were obtained from the herbal dealer Lukon Glads (Sadská, Czech Republic). The botanical identification was performed by Prof. L. Opletal. Voucher specimens are deposited in the Herbarium of the Faculty of Pharmacy in Hradec Králové under the following numbers: *Hippeastrum* cv. Pretty Nymph CUFPH-16130/AL-569, *Hippeastrum* cv. Artic Nymph CUFPH-16130/AL-574, *Hippeastrum* cv. Daphne CUFPH-16130/AL-563, *Hippeastrum* cv. Double King CUFPH-16130/AL-567, *Hippeastrum* x *hybridum* cv. Ferrari CUFPH-16130/AL-562, and *Hippeastrum* cv. Spartacus CUFPH-16130/AL-570.

4.4 Preparation of alkaloidal extract of *Hippeastrum* cultivars

Fresh bulbs (3 x 15 g) were extracted 3 times with EtOH (50 mL) at room temperature for 24 h. The solvent was evaporated under reduced pressure and the residue dissolved in 2% HCl (10 mL). After removal of neutral compounds with diethyl ether (3 x 15 mL), the extract was basified with 10% $NaHCO_3$, and the alkaloids extracted with EtOAc (3 x 15 mL). The organic solvent was removed by evaporation. The dry alkaloid fraction (5 mg) was dissolved in MeOH to a final concentration of 1 mg/mL for further analysis.

4.5 GC/MS analysis of an alkaloidal extract of *Hippeastrum* cultivars

GC/MS analysis was performed using an Agilent 7890A GC 5975 gas chromatograph coupled with MSD triple axis mass spectrometer Agilent 5975C operating in electron ionisation mode at 70 eV (Agilent Technologies, Santa Clara, California, USA). The separation itself was carried out on a HP-5MS column (30 m × 0.25 mm o.d. with 0.25 µm film) (Agilent Technologies, Santa Clara, California, USA). The temperature gradient program was set as follows: 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 inlet temperature was set to 280°C. 1 µl of alkaloid solution was injected (concentration 1 mg·ml^{-1}) in split mode (split ratio 1:10).

GC/MS analysis of the prepared alkaloidal extracts was made within the scope of the screening study of plants from the Amaryllidaceae family conducted in our laboratory [123-125].

4.6 Isolation of alkaloids from *Hippeastrum* cultivars

Using preparative TLC, five Amaryllidaceae alkaloids have been isolated in pure form from various *Hippeastrum* cultivars as a following:

4.6.1 Montanine: (25 mg) was isolated from the alkaloidal extract of *Hippeastrum* cv. Pretty Nymph (265 g, 187 mg of extract) by preparative TLC (To:Et_2NH 9:1, three times). The structure was determined by comparison of its MS and NMR data, and additional physical properties with literature data. The purity of the isolated compound was ≥ 95 %.

4.6.2 Vittatine: (12 mg) was isolated from the alkaloidal extract of *Hippeastrum* cv. Double King (191 g, 120 mg of extract) by preparative TLC (To:Et_2NH 9:1, two times). The structure was determined by comparison of its MS and NMR data, and additional physical properties with literature data. The purity of the isolated compound was ≥ 95 %.

4.6.3 11-Hydroxyvittatine: (12 mg) was isolated from the alkaloidal extract of *Hippeastrum* x *hybridum* cv. Ferrari (218 g, 120 mg of extract) by preparative TLC (To:cHx:Et_2NH 45:50:5, three times). The structure was determined by comparison of its MS and NMR data, and additional physical properties with literature data. The purity of the isolated compound was ≥ 95 %.

4.6.4 Lycorine: was isolated from the alkaloidal extract of *Hippeastrum* cv. Artic Nymph (256 g, 187 mg of extract) by preparative TLC (To:EtOH:Et_2NH 7:2:1, two times). The structure was determined by comparison of its MS and NMR data, and additional physical properties with literature data [126]. The purity of the isolated compound was ≥ 95 %.

4.6.5 Hippeastrine: (15 mg) was isolated from the alkaloidal extract of *Hippeastrum* cv. Daphne (175 g, 162 mg of extract) by preparative TLC (To:EtOH:Et_2NH 7:2:1, two times). The structure was determined by comparison of its MS and NMR data, and additional physical properties with literature data [127]. The purity of the isolated compound was ≥ 95 %.

4.7 Extraction and isolation of alkaloids from *Hippeastrum* x *hybridum* cv. Ferrari

Fresh bulbs (25 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 1.5-2 with 2% hydrochloric acid (HCl; 2.5 L), filtered. 900 ml of filtered extract alkalized to pH 9-10 with 190 ml of 10% Na_2CO_3 followed by extraction with $CHCl_3$ (4× 350 ml). The chloroformic extract was diluted with 1 L of HCl followed by defatted by extraction with ether (4× 300 ml). The water phase was subjected to alkalinization by the addition of 300 ml of 10% Na_2CO_3 and then extracted

with $CHCl_3$ (6×300 ml). The organic layer was evaporated to give 29.46 g of fluid residue. The obtained extract, which was Dragendorff positive, was further fractionated by CC on Al_2O_3 (1200 g), eluting with $CHCl_3$ gradually enriched with EtOH (99:1 – 0:100). Fractions of 500 mL were collected and monitored by TLC, yielding 360 fractions, which were combined into 21 fractions, followed by GC-MS analysis of each subfraction. Fractions with similar profiles were combined to give fifteen final fractions (**I-XV**) for further separation (Table 8, Fig. 12). The detailed process of the column chromatography is summarized in the following table (Table 8).

Table 7. The conditions of the column chromatography of the alkaloid extract

Weight of the summary extract	29.46 g
Adsorbent for the slurry	100 g
Adsorbent	Al_2O_3; 1200 g
Dead volume	1200 ml
Fractions	500 ml
Dividing layer in the column	112 cm
Layer with the alkaloid fraction	15 cm

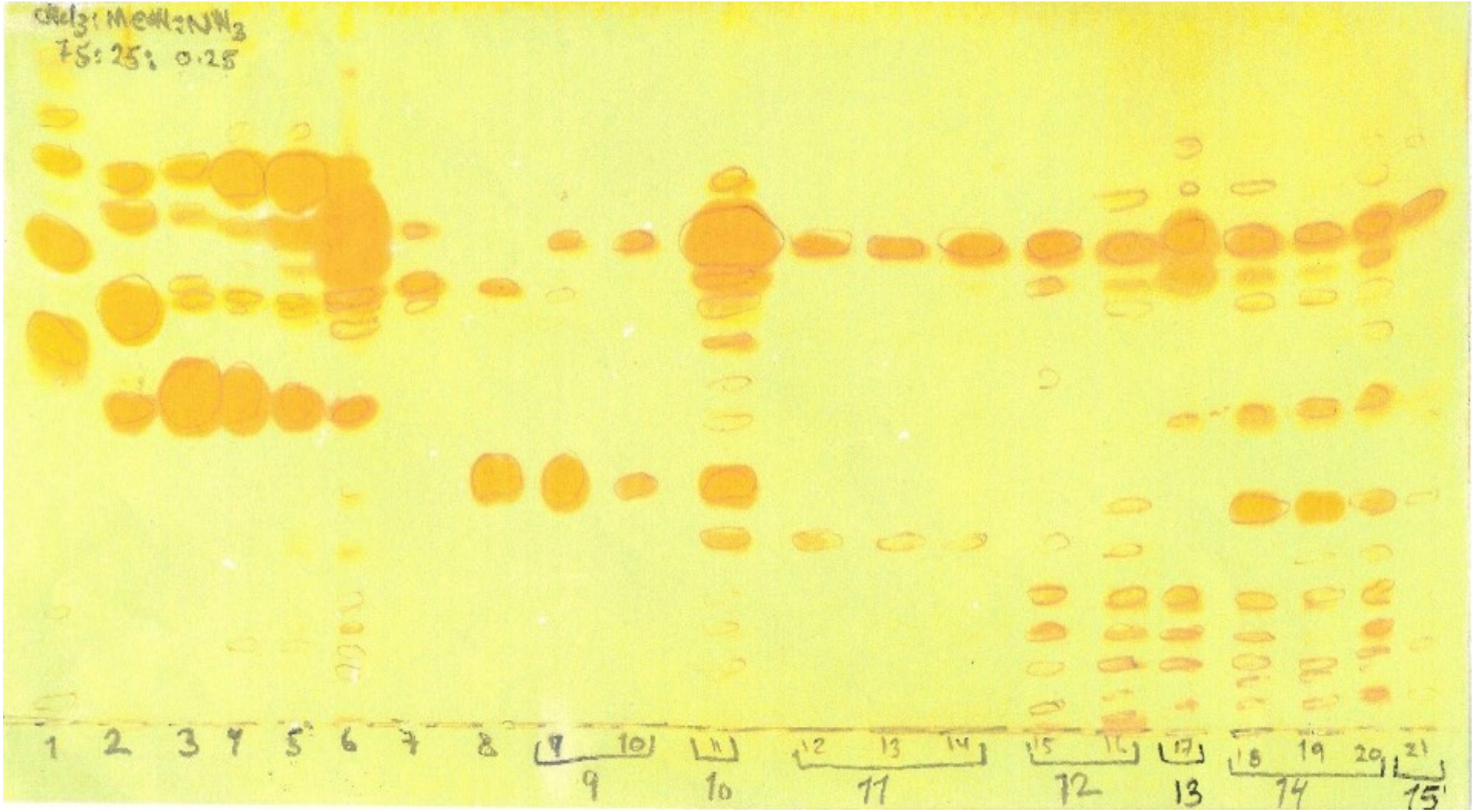


Fig. 12. TLC analysis of the final fractions obtained after column chromatography of alkaloidal extract of *Hippeastrum x hybridum* cv Ferrari

Table 8. The result of the column chromatography

Sign	Joined fractions	Composition of eluent	Weight (g)	Description
I	Fr. 1-17	CHCl3 (100)	1.93	brown, viscid
II	Fr. 18-29	CHCl3 (100)	2.91	brown, viscid
III	Fr. 30-56	CHCl3 (100)	3.16	brown, viscid
IV	Fr. 57-60	CHCl3:EtOH (99:1)	0.20	russet, viscid
V	Fr. 61-64	CHCl3:EtOH (99:1)	0.16	russet, crystals
VI	Fr. 65-72	CHCl3:EtOH (99:1)	0.32	brown, viscid
VII	Fr. 73-76	CHCl3:EtOH (99:1)	0.48	white precipitate
VIII	Fr. 77-87	CHCl3:EtOH (99:1)	3.91	white precipitate
IX	Fr. 88-101	CHCl3:EtOH (99:1)	1.65	white precipitate
X	Fr. 102-136	CHCl3:EtOH (99:1) CHCl3:EtOH (98:2)	1.41	brown, viscid
XI	Fr. 137-194	CHCl3:EtOH (98:2) CHCl3:EtOH (95:5)	1.32	brown, viscid
XII	Fr. 195-319	CHCl3:EtOH (95:5) CHCl3:EtOH (90:10) CHCl3:EtOH (85:15) CHCl3:EtOH (80:20) CHCl3:EtOH (75:25) CHCl3:EtOH (70:30) CHCl3:EtOH (65:35) CHCl3:EtOH (60:40) CHCl3:EtOH (55:45) CHCl3:EtOH (50:50)	1.25	brown, viscid
XIII	Fr. 320-332	CHCl3:EtOH (50:50) CHCl3:EtOH (45:55)	0.26	brown, viscid
XIV	Fr. 333-355	CHCl3:EtOH (35:65) CHCl3:EtOH (25:75) EtOH (100)	0.67	brown, viscid
XV	Fr. 355-360	MeOH:acetic acid (98:2)	2.62	brown, viscid

Fraction I (1.93 g) was processed using preparative TLC with the use of the mobile phase **MP-1**. The plates were developed three times to give six subfractions (**I/1-6**). Subfraction **I/2** was chromatographed using preparative TLC with the use of the mobile phase **MP-2**. The plates were developed in the mobile phase once. Six zones were obtained (**I/2/1-6)**. The zone **I-2/3** was further treated by preparative TLC using mobile phase **MP-2**. Five zones (**I/2/3A-E**) have been obtained. The zone **I-2/3C** gave after recrystallization brown powder of dihydro-2-*O*-acetyllycorine (**HF-1**, 150 mg). The zone **I/2/4** was further treated by preparative TLC using the mobile phase **MP-2** to give four zones **I/2/4A-D**. Subfraction **I/2/4C** gave after preparative TLC with the use of the mobile phase **MP-2** brown powder of alkaloid montanine (**HF-2**, 5.1 mg). The zone **I/3** was recrystallized ($CHCl_3$:EtOH 1:1) to give white crystals of haemanthamine (**HF-3**, 700 mg). The zone **I/4** was processed via preparative TLC with the use of the mobile phase **MP-3**. The plates were developed in the used mobile phase once. Four fractions were obtained (**I/4/1-4**). The zone **I-4/3** gave after recrystallization white crystals of tazettine (**HF-4**, 161.2 mg). The zone **I/4/4** was eluted with $CHCl_3$:EtOH in the ratio 1:1, and 12 mg of 1, 2-*O*,*O*'-diacetyl-dihydrolycorine was obtained (**HF-5**).

Fraction **I/6** was further fractionated by preparative TLC using the mobile phase **MP-4**. Three subfractions were obtained **I/6/1-3**. Subfraction **I/6/1** gave ismine (**HF-6**, 25 mg), and subfraction **I/6/3** gave 3-epimacronine (**HF-7**, 7 mg). The remaining subfractions were not used for further separation due to the low amount.

Fraction II (2.9 g) was subjected to preparative TLC using mobile phase **MP-5** to give an additional amount of montanine (1.8 g), and tazettine (181 mg).

Fraction III (3.16 g) was crystallized from EtOH yielding 2.1 g of vittatine (**HF-8**).

Fraction IV (0.2 g) was subjected to preparative TLC using the mobile phase **MP-6**. The plates were developed in the mobile phase once. Six zones (**IV/1-6**) were obtained. The zone **IV/2** yield again vittatine (6 mg), while zone **IV/5** was subjected to preparative TLC using **MP-5** to give an additional amount of tazettine (38.8 mg). The remaining subfractions were not further separated due to the number of minor alkaloids and low amount.

Fraction V (0.16 g) was subjected to preparative TLC using the mobile phase **MP-5**. The plates were developed in the mobile phase once. Seven zones (**V/1-7**) were obtained. The zone **V/3** gave after recrystallization alkaloid crinine (**HF-9**, 20 mg), while zone **V/6** was subjected to preparative TLC using **MP-5** to give an additional amount of tazettine (6 mg). The remaining subfractions were not further separated due to the number of minor alkaloids, and low amount.

Fraction VI (0.32 g) was crystallized from EtOH yielding lycorine (**HF-10**, 22.3 mg). The mother liquor of fraction **VI** was further treated by preparative TLC using the mobile phase **MP-5**. The plates were developed in the mobile phase once. Five subfractions were obtained (**VI/1-5**). Subfraction **VI/2** gave an additional amount of tazettine (45 mg).

Fraction VII (0.48 g) was crystallized from EtOH to give an additional amount of lycorine (250 mg).

Fraction VIII (3.91 g) was subjected to preparative TLC using the mobile phase **MP-6**. The plates were developed in the mobile phase once. Two zones (**VIII/1-2**) were obtained. The zone **VIII/1** yield again lycorine (1.52 g), while zone **VIII/2** was recrystallized with EtOH to give white crystals of 11-hydroxyvittatine (**HF-11**, 1.04 g).

Fraction IX (1.65 g) was crystallized from EtOH to give an additional amount of 11-hydroxyvittatine (529 mg). The mother liquor of fraction **IX** was further treated by preparative TLC using the mobile phase **MP-6**. Three zones were obtained (**IX/1-3)**. The zone **IX/1** was recrystallized ($CHCl_3$:EtOH 1:1) to give white powder of homolycorine (**HF-12**, 320 mg).

Fraction X (1.41 g) was further separated by preparative TLC using the mobile phase **MP-9**. The plates were developed in the mobile phase twice to give seven subfractions (**X/1-7**). Repeated preparative TLC of fraction **X/4** using mobile phase **MP-9** gave four zones (**X/4/1-4**).

Subfraction **X/4/4** gave an additional amount of homolycorine (18 mg). Subfraction **X/5** was subjected to additional preparative TLC using mobile phase **MP-6** to give again 11-hydroxyvittatine (1.8 g).

Fraction XI (1.32 g) was crystallized from EtOH yielding 30 mg of pancracine (**HF-13**). The mother liquor of fraction **XI** was further treated by preparative TLC using the mobile phase **MP-6** to give six subfractions (**XI/1-6**). Subfraction **XI/I** was recrystallized from EtOH to give dihydrolycorine (**HF-14**, 2 mg). Subfraction **XI/5** was treated by preparative TLC using mobile phase **MP-7** to give four zones (**XI/5/1-4**). Subfraction **XI/5/1** gave 9-*O*-demethylhomolycorine (**HF-15**, 290 mg). The remaining subfractions were not further separated due to the high number of minor alkaloids and low amount.

Fraction XII (1.25 g) was subjected to preparative TLC using the mobile phase **MP-6**. The plates were developed in the mobile phase once. Two zones (**XII/1-2**) were obtained. Additional preparative TLC of subfraction **XII/I** using the mobile phase **MP-5** to give seven fractions (**XII/1/1-7**). Subfraction **XII/1/5** gave after repeated preparative TLC zephyranthine (**HF-16**, 29 mg). The remaining subfractions were not used for further separation due to the low amount.

Fractions XIII (0.26 g) was processed using preparative TLC with the use of the mobile phase **MP-10**. The plates were developed in the mobile phase once. Six subfractions (**XIII/1-6**) were obtained. Subfraction **XIII/2** gave an additional amount of 9-*O*-demethylhomolycorine (65 mg). The subfraction **XIII/1** was further treated by preparative TLC using mobile phase **MP-5**. Two zones (**XIII/1/1-2**) have been obtained. The zone **XIII/1/1** gave again an additional amount of vittatine (6 mg).

Fraction XIV (0.67 g) was processed using preparative TLC with the use of the mobile phase **MP-11, MP-12,** and **MP-13,** the plates were developed once. Five subfractions (**XIV /1-5**). Subfraction **XIV /2** gave an additional amount (300 mg) of 11-hydroxyvittatine while subfraction **XIV /5** gave additional tazettine (120 mg).

Fraction XV (2.62 g) was fractionated by preparative TLC using the mobile phase **MP-10**. The plates were developed in the mobile phase two times. Ten subfractions (**XV/1-15**) were obtained. Zone **XV/4** was recrystallized from EtOH to give 9-*O*-demethyl-7-*O*-methyllycorenine (**HF-17**, 8.1 mg). The zone **XV/5** was subjected to additional preparative TLC using the mobile phase **MP-10**. The plates were developed in the mobile phase once to give 9-*O*-demethyllycorenine (**HF-18**, 12 mg). Further subfractions were not used for separation due to the complex mixtures and low amounts for separation.

4.8 Structural identification of isolated alkaloids

4.8.1 Mass spectrometry with electrospray ionisation

For compounds that could not be analyzed due to their low volatility, thermal instability, and problematic ionization on GC/MS with electron ionization, liquid chromatography coupled with mass spectrometry was used. The analysis was performed on the Waters Autopurification™ HPLC-MS system (Waters Corporation, Milford, USA). The liquid chromatograph consisted of an autosampler with fraction collector - Waters Sample Manager 2767, with an injection option in the range of 1 - 20 µl, equipped with a 1 ml injection loop. The instrument included a column selector - System Fluidics Organizer with the possibility of switching between 3 analytical and 3 preparative columns. A binary gradient pump Waters 2545, which operates in the range of 0.5 to 150 ml/min, and a maximum pressure of 42 MPa was employed. For detection, the instrument uses a Waters 2998 diode array detector with an absorbance collection in the range of 190 to 800 nm and a Waters Acquity qDa mass spectrometer, capable of collecting data in the range of 30 to 1250 m / z equipped with electrospray ionization. Specimens (0.2 – 0.3 mg) were dissolved in 1 mL of LC-MS grade methanol. The sample was analyzed at ambient temperature using XSelect® CSH™ C18 OBD™ reverse phase column (100 mm × 4.6 mm i.d., 5 µm) (Waters Corporation, Milford, USA). Water with 0.1% formic acid (solvent A) and Methanol with 0.1% formic acid (solvent B) were used as mobile phases. The flow rate of the mobile phase was 1 mL/min. The gradient elution program was programmed as follows (v/v): 0 min 5% B, 5 min 100% B, 8.5 min 5% B, followed by 1.5 min at initial conditions for re-equilibration. The optimum values of the ESI-MS parameters were: capillary voltage – 0.8 kV; Probe temperature - 600°C; Cone Voltage - 15V. LC/MS mass spectra were recorded across the range of 200–800 m/z. For PDA detection the detector range was set from 190 to 700 nm. LC ESI-MS analyses were carried out in the positive ion mode. The elution of compounds occurs in order from more polar to less polar, driven by the increasing elution strength of the mobile phase. Throughout the chromatography, the concentration of formic acid remains constant.

4.8.2 Mass spectrometry with electron ionization

The same procedure as described previously (Chapter 4.5) has been used.

4.8.3 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.

4.8.4 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 $CDCl_3$ and CD_3OD solutions, respectively, 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 the 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; for CD_3OD: = 3.30 ppm for ^{1}H atoms and δ= 49.0 ppm for ^{13}C atoms. The data required by measurements are introduced in the order: chemical shift (δ), the integrated intensity of ^{1}H NMR spectrum, multiplicity (s: singlet, d: doublet, dd: doublet of doublets, ddd: doublet of doublet of doublets, ddt: doublet of doublet of triplets, dt: doublet of triplets, t: triplet, q: quartet, m: multiplet) and the interaction constant *J* (Hz).

The two-dimensional (2D) NMR experiments (gCOSY, gHSQC, gH2BC, gHMBCAD, 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 cross peak appears between protons that 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 ^{1}H and ^{13}C through one bond.

gH2BC (gradient heteronuclear two-bond correlation): a two-dimensional NMR technique that helps to specify a correlation of ^{1}H with ^{13}C through two bonds.

gHMBCAD (adiabatic gradient heteronuclear multiple bond coherence): a two-dimensional NMR technique that helps to specify a correlation of ^{1}H with ^{13}C through three bonds (in some cases even 2 or 4 bonds).

NOESY (nuclear Overhauser effect spectroscopy): a two-dimensional NMR technique where cross peaks appear between protons that are remote one from another to 5 Å. The intensity of the cross peak drops with the more remote distance of the protons in the area.

4.8.5 Optical rotation

The optical rotation of the alkaloids was measured in $CHCl_3$ solution on a P3000 polarimeter. Specific rotation was calculated according to the formula:

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

t – temperature of the measurements; D – line of natrium light λ = 589.3 nm; α – measured rotation [°]; c – concentration of the measured alkaloid [g·100 ml-1]; $^{l-}$ length of the cell [dm].

4.8.6 Circular dichroism

ECD spectra were recorded on a JASCO J-815CD spectrometer.

4.9 Biological assays of isolated alkaloids

4.9.1 *In vitro* Anti-Cholinesterase Assay

The AChE and BChE inhibitory activity of the tested compounds was determined using a modified Ellman method [128]. Human recombinant acetylcholinesterase (*h*AChE; EC 3.1.1.7, human plasma butyrylcholinesterase (*h*BChE; EC 3.1.1.8), 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent, DTNB), phosphate buffer (PB, pH 7.4), acetylthiocholine (ATCh), and butyrylthiocholine (BTCh), were purchased from Sigma-Aldrich (Prague, Czech Republic). For measuring purposes–polystyrene Nunc96-well microplates with flat bottom shape (Thermo Fisher Scientific, Waltham, MA USA) were utilized. All the assays were carried out in 0.1 M KH_2PO_4/K_2HPO_4 buffer, pH 7.4. Enzyme solutions were prepared at activity 2.0 units/mL in 2 mL aliquots. The assay medium (100 μL) consisted of 40μL of 0.1 M phosphate buffer (pH 7.4), 20 μL of 0.01 M DTNB, 10 μL of the enzyme, and 20 μL of 0.01 M substrate (ATCh iodide solution).

Assay solutions with inhibitor (10 μL, 10^{-3}-10^{-9} M) were preincubated for 5 min. The reaction was started by the addition of 20 μL of the substrate (ATCh for *h*AChE, BTCh for *h*BChE). The enzyme activity was determined by measuring the increase in absorbance at 412 nm at 37 ºC at 2 min intervals using a multi-mode Synergy 2 microplate reader (Bio-Tek, Winooski, VT, USA). Each concentration was assayed in triplicate. The obtained data were used to compute the percentage of inhibition (I, Equation (1)):

$$I = \left(1 - \frac{\Delta A_i}{\Delta A_0}\right) \times 100\ [\%]$$

ΔA_i indicates absorbance change provided by cholinesterase exposed to AChE inhibitors and ΔA_0 indicates absorbance change caused by intact cholinesterase (phosphate buffer was used instead of AChE inhibitor solution). Inhibition potency of tested compounds was expressed as

IC_{50} value(concentration of inhibitor, which causes 50% cholinesterase inhibition). Calculations were performed using the Microsoft Excel software (Microsoft Inc., Redmond, WA, USA) and GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA) [129].

4.9.2 Prolyloligopeptidase assay

Pop inhibition activity has been measured in cooperation with the Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, University of Defence. Detailed can be found in our previous report [130].

4.9.3 *In vitro* cytotoxicity study

Cytotoxic activity has been measured in cooperation with the Department of Medical Biochemistry, Faculty of Medicine in Hradec Králové, Charles University.

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 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 an exponential growth phase were used for this study.

Cell treatment

All the alkaloids evaluated and doxorubicin, used as a positive control, were dissolved in dimethyl sulfoxide – DMSO (Sigma-Aldrich, St. Louis, USA) to prepare stock solutions with a concentration of 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% CO_2 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: (%) viability = $(A_{sample} - A_{blank}) / (A_{control} - A_{blank}) \times 100$, where A is the absorbance of the employed WST-1 formazan measured at 440 nm [131]. 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 vehicle control.

Statistical analysis

The descriptive statistics of the results were calculated and the charts were 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.

5 RESULTS

5.1 GC/MS analysis of *Hippeastrum* cultivars

In the bulb extracts of the studied six ornamental varieties of *Hippeastrum* cultivars, 20 compounds with typical mass spectra of Amaryllidaceae alkaloids were detected. Eighteen of them were identified based on their mass spectra, retention times, and retention indexes. Identified alkaloids belong to the crinine, haemanthamine, galanthamine, homolycorine, lycorine, montanine, and tazettine structural types of Amaryllidaceae alkaloids. The alkaloids marked as A1, A2, and A3 displayed mass spectra typical for Amaryllidaceae alkaloids, however, left unidentified. Considering their low concentrations (< 5% of TIC), isolation and structural elucidation of them could be problematic. From the mass spectrum of alkaloid A2, some structural aspects can be concluded. The fragmentation pattern, especially the presence of an intense peak at m/z 109 such as in masonine and homolycorine [132], indicates the homolycorine structural-type of Amaryllidaceae alkaloids. Other fragments of the mass spectrum displayed only weak intensities, and are not valuable for the exact identification of detected alkaloids. The relative proportion of each alkaloid was determined as a percentage of the total ion current (TIC). The peak areas reflect the ability of each compound to be ionized and thus the data given in Table 9 are semiquantitative. Nevertheless, they can be used for comparison between samples. Furthermore, five Amaryllidaceae alkaloids from different *Hippeastrum* cultivars have been isolated by preparative TLC in pure form.

Based on the obtained GC/MS results it can be concluded that cultivars of *Hippeastrum* are a rich source of different biologically active Amaryllidaceae alkaloids (Table 9). Lycorine, the most abundant Amaryllidaceae alkaloid, has been identified in all the studied cultivars, with the highest concentration being detected in *Hippeastrum* cv. Daphne (56% of TIC). In fresh bulbs, galanthamine, the most well known Amaryllidaceae alkaloid, was detected, but only in some cultivars, and mostly in trace concentration. In the alkaloidal extract of *Hippeastrum*. Cv. Pretty Nymph, two alkaloids of the homolycorine structural type have been identified as major components, namely homolycorine (40% of TIC) and hippeastrine (22 % of TIC).

Using preparative TLC, five Amaryllidaceae alkaloids have been isolated in pure form from various *Hippeastrum* cultivars (*Hippestrum* cv. Pretty Nymph, *Hippeastrum* cv. Double King, *Hippeastrum* x *hybridum* cv. Ferrari, *Hippeastrum* cv. Artic Nymph, *Hippeastrum* cv. Daphne). The compounds were identified by MS, 1D, and 2D NMR spectroscopic analyses and

by comparison of the obtained data with the literature as montanine, vittatine, 11-hydroxyvittatine, lycorine, and hippeastrine respectively.

Table 9. Composition of the alkaloidal extracts of six *Hippeastrum* cultivars analyzed by GC/MS

Alkaloid	**RI**	**Liter. Values of RI**	***Hippeastrum* x *hybridum* cv. Ferrari**	***Hippeastrum* cv. Double King**	***Hippeastrum* cv. Daphne**	***Hippeastrum* cv. Artic Nymph**	***Hippeastrum* cv. Pretty Nymph**	***Hippeastrum* cv. Spartacus**	**Ref. For MS, RI data**
Ismine	2278	2280	t			t			[133]
Trisphaeridine	2284	2282	t						[134]
Galanthamine	2408	2410			t	t		t	c.d, [135]
Lycoramine	2442	2417		<1	t				c,d, [136]
Vittatine/Crinine*	2498	2472	9	9	<1	4		3	C,d, [133]
A1	2518	n.a.			3				
9-*O*-Demethyllycosinine B	2575	2499	t	<1					[106]
11.12-Dehydroanhydrolycorine	2604	2606	<1		t				[133; 135]
A2 Homolycorine	2609	n.a.		3	<1				
Montanine	2615	2611	6	16			21	12	c,d, [133]
Haemanthamine	2640	2641	3	4	3	4		8	c,d, [133]
Tazettine/Pretazettine*	2655	2653	5		t			43	c,d, [133]
Pancracine	2719	2718	7	6			3		c,d, [133]
11-Hydroxyvittatine	2736	2732	49	23		18		8	c, [135]
Lycorine	2749	2746	9	30	56	44	15	26	c,d, [133]
Homolycorine	2769	2767	5	2			40		c, [134]
3-Epimacronine	2813	2811	t						c,d, [133]
Pseudolycorine	2823	2831	4	2	3	15			[135; 136]
Hippeastrine	2918	2917	1	3	34	5	22	t	c, [133]
A3	3012	n.a.				4	t		

*Cannot be distinguished by GC/MS; [a]For GC condition see Experimental section; [b]Values are expressed as a percentage of the total ion current (TIC); [C]standard; [d]NIST 11; t stands for trace, n.a. stands for not available

The cultivated cultivar *Hippeastrum* x *hybridum* cv. Ferrari was chosen for detailed phytochemical and biological studies because of its widest spectra of alkaloids which seemed to be promising compounds, and which could be isolated in sufficient amounts allowing us to perform a wide range of biological study optionally, the preparation of the semisynthetic derivatives. Fig. 13. shows the chromatogram of the summary alkaloid extract from the bulbs of *Hippeastrum* x *hybridum* cv. Ferrari, along with some identified alkaloids.

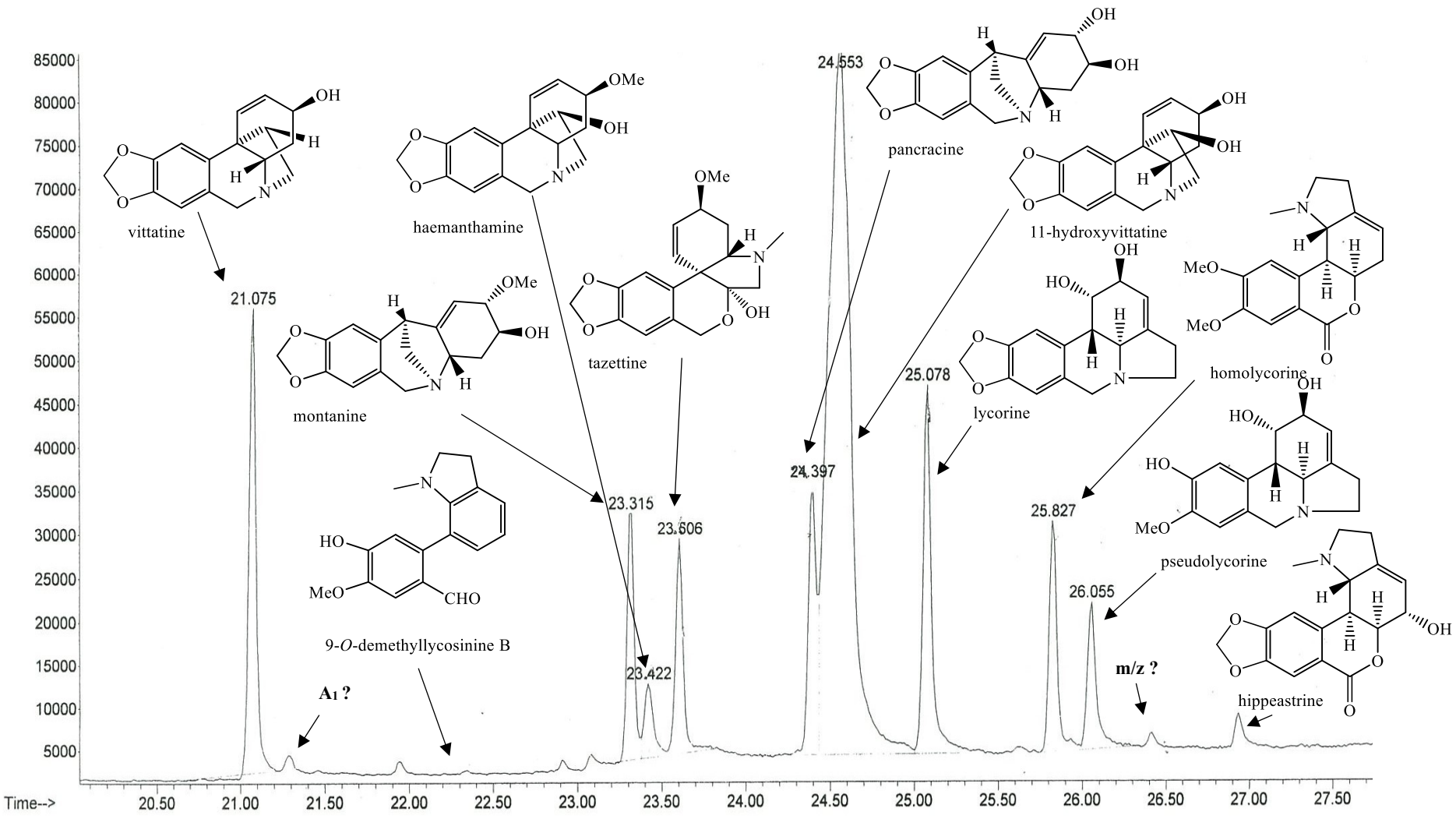


Fig. 13. GC/MS chromatogram of alkaloidal extract from fresh bulbs of *Hippeastrum* x *hybridum* cv. Ferrari

5.2 The overview of the isolated alkaloids from *Hippeastrum* x *hybridum* cv. Ferrari and their structural analysis

5.2.1 HF-1: Dihydro-2-*O*-acetyllycorine

Summary formula: $C_{18}H_{21}NO_5$

Based on the performed experiments (MS, NMR, optical rotation) and the comparison of the obtained data with the literature, the compound was identified as the lycorine-type alkaloid dihydro-2-*O*-acetyllycorine. This compound has been isolated as a mixture of C2-epimers, 1:3 ratio (A:B) (Fig. 14).

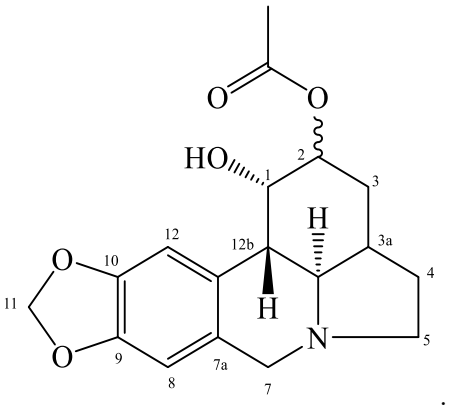


Fig. 14. Dihydro-2-*O*-acetyllycorine

Molecular weight: 331.37

MS analysis

EIMS *m/z* (%): 331(46), 330(100), 288(7), 270(77), 254(31), 213(15), 147(14).

NMR analysis

^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.86 (s, 1H, isomer A), 6.66 (s, 1H, isomer B), 6.61 (s, 1H, isomer A), 6.60 (s, 1H, isomer B), 5.95 – 5.89 (m, 4H, isomer A + isomer B), 5.11 (ddd, *J* = 11.9 Hz, *J* = 5.4 Hz, *J* = 2.5 Hz, 1H, isomer A), 4.72 – 4.68 (m, 2H, isomer A + isomer B), 4.30 (d, overlap, *J* = 16.5 Hz, 1H, isomer A), 4.29 (d, overlap, *J* = 16.5 Hz, 1H, isomer B), 4.14 (ddd, *J* = 10.0 Hz, *J* = 6.3 Hz, *J* = 2.7 Hz, 1H, isomer B), 3.78 – 3.70 (m, 2H, isomer A + isomer B), 3.47 – 3.32 (m, 2H, isomer A+ isomer B), 3.22 (dd, *J* = 10.5 Hz, *J* = 6.0 Hz, 1H, isomer A), 3.11 (dd, *J* = 10.4 Hz, *J* = 5.9 Hz, 1H, isomer B), 2.86 – 2.76 (m, 2H, isomer A + isomer B), 2.73 – 2.64 (m, 2H, isomer A + isomer B), 2.63 (d, *J* = 10.5 Hz, 1H, isomer B), 2.55 (d, *J* = 10.5 Hz, 1H, isomer A), 2.22 (td, *J* = 12.7 Hz, *J* = 6.2 Hz, 1H, isomer A), 2.15 (s, 3H, isomer A), 2.08 (s, 3H, isomer B), 2.07 – 1.71 (m, 9H, isomer A + isomer B).

^{13}C NMR (126 MHz, $CDCl_3$) δ: 172.4 (isomer B), 170.4 (isomer A), 146.6 (isomer A), 146.5 (isomer B), 146.1 (isomer B), 146.1 (isomer B), 129.0 (isomer A + isomer B), 128.5 (isomer A), 128.3 (isomer B), 107.1 (isomer A), 107.0 (isomer B), 104.5 (isomer A), 104.1 (isomer B), 100.8 (isomer A + isomer B), 72.1 (isomer A), 68.6 (isomer B), 67.1 (isomer A + isomer B), 58.4 (isomer B), 58.3 (isomer A), 57.6 (isomer A + isomer B), 53.1 (isomer A), 52.9 (isomer B), 52.6 (isomer A), 52.4 (isomer B), 36.8 (isomer B), 36.6 (isomer A), 34.7 (isomer A), 33.6 (isomer B), 27.0 (isomer B), 26.7 (isomer A), 25.8 (isomer A + isomer B), 21.3 (isomer A), 21.0 (isomer B).

5.2.2 HF-2: Montanine

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

Based on the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the montanine-type alkaloid montanine (Fig. 15) [17].

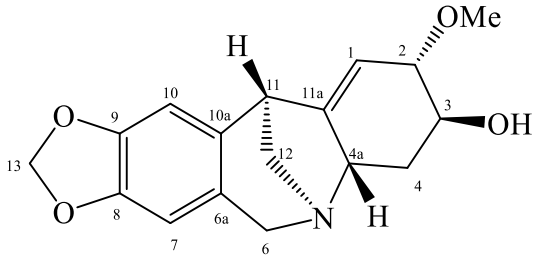


Fig. 15. Montanine

Molecular weight: 301.34

MS analysis

EIMS *m/z* (%): 301(100), 270(88), 257(35), 252(25), 229(28), 226(30), 223(30), 199(20), 185(35), 115(20).

NMR analysis

^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.56 (1H, s), 6.47 (1H, s), 5.91–5.90 (1H, m), 5.88–5.87 (1H, m), 5.60–5.58 (1H, m), 4.36 (1H, d, *J* = 16.6 Hz), 4.12–4.09 (1H, m), 3.84 (1H, d, *J* = 16.6 Hz), 3.50–3.48 (1H, m), 3.48–3.45 (2H, m, overlapped), 3.45 (3H, s, overlapped), 3.33–3.31 (1H, m), 3.11 (1H, dd, *J* = 11.3 Hz, *J* = 2.4 Hz), 3.05 (1H, d, *J* = 11.3 Hz), 2.24–2.17 (1H, m), 1.59 (1H, td, *J* = 12.2 Hz , *J* = 3.5 Hz).

^{13}C NMR (125 MHz, $CDCl_3$) δ: 153.5, 146.8, 146.1, 132.2, 124.2, 113.2, 107.3, 106.8, 100.8, 79.6, 68.8, 60.7, 58.8, 57.6, 55.3, 45.5, 32.5

Optical rotation

$[\alpha]^{25}_{D}$ = -87.6° (c = 0.57, $CHCl_3$).

5.2.3 HF-3: Haemanthamine

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

Based on 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. 16) [47].

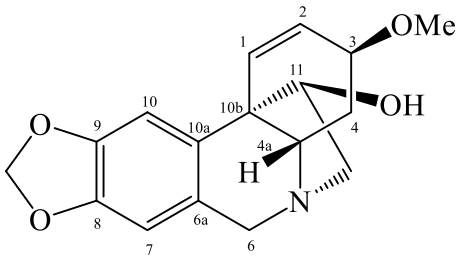


Fig. 16. Haemanthamine

Molecular weight: 301.34

MS analysis

EIMS *m/z* (%): 301(15), 272(100), 240(15), 225(5), 211(15), 128(10).

NMR analysis

^{1}H NMR (500 MHz, $CDCl_3$) δ: 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_2O), 5.76 (1H, s, OCH_2O), 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}C NMR (125 MHz, $CDCl_3$) δ: 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

$[\alpha]^{25}_{D}$ = +37° (c = 0.29, $CHCl_3$).

5.2.4 HF-4: Tazettine

Summary formula: $C_{18}H_{21}NO_5$

Based on 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. 17) [137] .

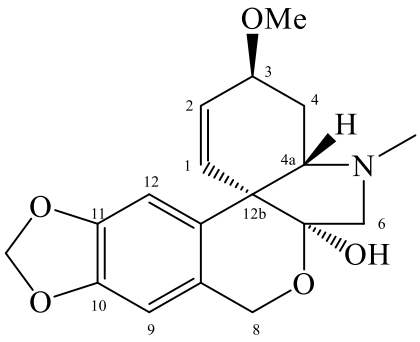


Fig. 17. Tazettine

Molecular weight: 331.36

MS analysis

EIMS *m/z* (%): 331(20), 316(20), 298(25), 247(100), 230(10), 201(15), 181(10), 152(8).

NMR analysis

^{1}H NMR (500 MHz, $CDCl_3$) δ: 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_2O), 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, $CDCl_3$) δ: 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

$[\alpha]^{25}_D$ = +164.0° (c = 0.1, MeOH).

5.2.5 HF-5: 1, 2-*O,O'*-Diacetyl-dihydrolycorine

Summary formula: $C_{20}H_{23}NO_6$

Based on 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 1,2-*O,O'*-diacetyl-dihydrolycorine (Fig. 18) [16].

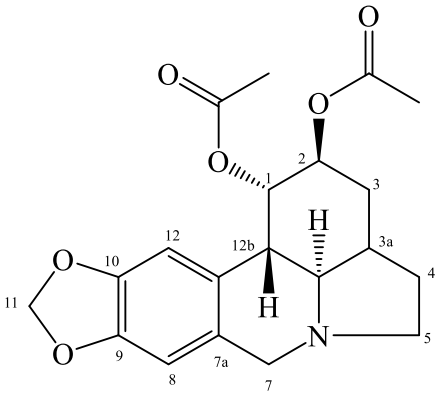


Fig. 18. 1, 2-*O,O'*-Diacetyl-dihydrolycorine

Molecular weight: 373.41

MS analysis

EIMS *m/z* (%): 374(10), 373(50), 372(100), 314(44), 272(29), 254(53), 252(31), 147(12).

NMR analysis

^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.63 (s, 1H), 6.58 (s, 1H), 6.06 – 6.03 (m, 1H), 5.90 (d, overlap, *J* = 4.9 Hz, 1H), 5.90 (d, overlap, *J* = 4.9 Hz, 1H), 5.15 (ddd, *J* = 12.0 Hz, *J* = 5.4 Hz, *J* = 2.6 Hz, 1H), 4.28 (d, *J* = 16.8 Hz, 1H), 3.75 (d, *J* = 16.8 Hz, 1H), 3.70 – 3.61 (m, 1H), 3.36 (td, *J* = 9.3 Hz, *J* = 6.0 Hz, 1H), 3.11 (dd, *J* = 10.2 Hz, *J* = 6.0 Hz, 1H), 2.79 (td, *J* = 10.2 Hz, *J* = 4.8 Hz, 1H), 2.74 – 2.65 (m, 2H), 2.16 (ddd, *J* = 13.5 Hz, *J* = 12.0 Hz, *J* = 6.0 Hz, 1H), 2.07 – 1.99 (m, overlap, 3H), 2.05 (s, *J* = 1.4 Hz, 3H), 1.94 – 1.88 (m, 1H), 1.87 – 1.76 (m, 1H).

^{13}C NMR (126 MHz, $CDCl_3$) δ: 170.8, 170.7, 146.4, 146.1, 128.5, 128.3, 106.9, 104.2, 100.8, 69.9, 67.5, 58.0, 52.7, 52.3, 36.8, 33.2, 26.6, 26.6, 21

Optical rotation

$[\alpha]^{25}_D$ = + 48 ° (c = 0.10, $CHCl_3$).

5.2.6 HF-6: Ismine

Summary formula: $C_{15}H_{15}NO_3$

The substance was identified as an alkaloid of the phenanthridine structural type ismine (Fig.19) [138].

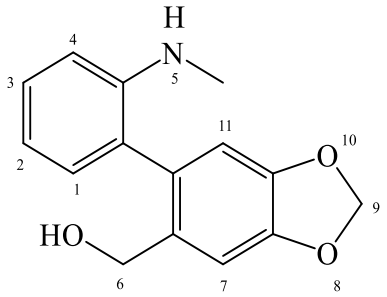


Fig. 19. Ismine

Molecular weight: 257.28

MS analysis

EIMS *m/z* (%): 257(28), 239(10), 238(100), 225(7), 211(7), 196(10), 180(8), 139(10).

NMR analysis

^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.28 (1H, ddd, *J* = 8 Hz, *J* =7 Hz, *J* = 2 Hz, H-3), 7.00 (1H, s, H-10), 6.98 (1H, s, H-1), 6.81 (1H, ddd, *J* = 7 Hz, *J* = 7 Hz, *J* = 1 Hz, H-2), 6.73 (1H, dd, *J* = 8, *J* = 1 Hz, H-4), 6.67 (1H, s, H-7), 5.99 (2H, s, OCH_2O), 4.26 (1H, d, *J* = 12 Hz, H-6), 4.20 (1H, d, *J* = 12 Hz, H-6), 2.73 (3H, s, NCH_3).

^{13}C NMR (125 MHz, CDCl3) δ: 147.5 (C-8), 147.4 (C-9), 146.7 (C-4a), 134.0 (C-6a), 131.2 (C-10a), 129.9 (C-1), 129.1 (C-3), 127.2 (C-10b), 118.0 (C-2), 110.7 (C-4), 110.2 (C-10), 109.7 (C-7), 101.3 (OCH_2O), 63.5 (C-6), 30.8 (NCH_3).

Optical rotation

This substance does not have a chiral center, the optical rotatory has not been measured.

5.2.7 HF-7: 3-Epimacronine

Summary formula: $C_{18}H_{19}NO_5$

Based on 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 3-epimacronine (Fig. 20) [139].

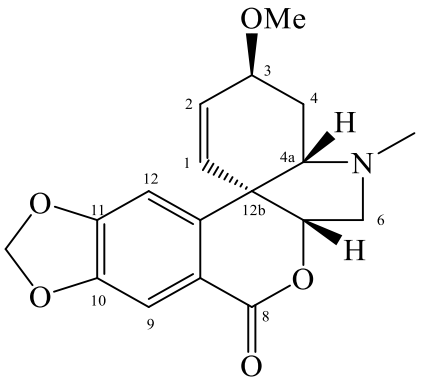


Fig. 20. 3-Epimacronine

Molecular weight: 329.35

MS analysis

EIMS *m/z* (%): 329(30), 314(25), 245(100), 225(15), 201(80), 139(10).

NMR analysis

^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.53 (s, 1H), 6.76 (s, 1H), 6.05 (d, *J* = 10.4 Hz, 1H), 5.98 (d, overlap, *J* = 10.4 Hz, 1H), 5.98 (d, overlap, *J* = 10.4 Hz, 1H), 5.49 (dt, *J* = 10.4 Hz, *J* = 1.9 Hz, 1H), 4.47 (dd, *J* = 11.0 Hz, *J* = 7.8 Hz, 1H), 4.19 – 4.14 (m, 1H), 3.44 (s, 3H), 3.20 (dd, *J* = 11.1 Hz, *J* = 10.0 Hz, 1H), 3.15 – 3.12 (m, 1H), 2.81 (dd, *J* = 10.0 Hz, *J* = 7.8 Hz, 1H), 2.59 – 2.54 (m, overlap, 1H), 2.53 (s, overlap, 3H), 1.73 (ddd, *J* = 13.4 Hz, *J* = 10.0 Hz, *J* = 2.1 Hz, 1H).

^{13}C NMR (126 MHz, $CDCl_3$)δ: 165.6, 152.3, 147.1, 142.2, 131.3, 126.1, 118.6, 111.0, 103.8, 102.1, 80.1, 72.8, 63.3, 56.2, 53.5, 46.2, 42.9, 29.8

Optical rotation

$[\alpha]^{28}_D$ = +225.6° (c = 0.15, $CHCl_3$).

5.2.8 HF-8: Vittatine

Summary formula: $C_{16}H_{17}NO_3$

Based on 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 vittatine (Fig. 21) [140].

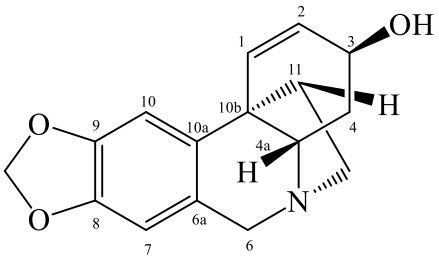


Fig. 21. Vittatine

Molecular weight: 271.31

MS analysis

EIMS *m/z* (%): 271(100), 228(25), 199(90), 187(80), 173(30), 128(30), 115(35), 56(20).

NMR analysis

^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.85 (1H, s), 6.59 (1H, d, *J* = 9.8 Hz), 6.47 (1H, s), 5.67 (1H, dd, *J* = 9.8 Hz, *J* = 5.3 Hz), 5.90 (1H, d, *J* = 8.3 Hz, overlapped), 5.89 (1H, d, *J* = 8.3 Hz, overlapped), 4.37 (1H, d, *J* = 17.0 Hz, overlapped), 4.36–4.33 (1H, m, overlapped), 3.76 (1H, d, *J* = 17.0 Hz), 3.41–3.31 (2H, m), 2.93–2.85 (1H, m), 2.18 (1H, ddd, *J* = 12.2 Hz, *J* = 9.3 Hz, *J* = 4.4 Hz), 2.02–1.96 (1H, m), 1.91 (1H, ddd, *J* = 12.2 Hz, *J* = 10.3 Hz, *J* = 5.8 Hz), 1.74 (1H, td, *J* = 13.7 Hz, *J* = 4.4 Hz).

^{13}C NMR (125 MHz, $CDCl_3$) δ: 146.1, 145.7, 138.4, 132.2, 127.5, 126.4, 107.0, 102.8, 100.7, 64.0, 62.8, 62.4, 53.6, 44.25, 44.22, 32.8

Optical rotation

$[\alpha]^{25}_D$ = + 47.5° (c = 0.152, $CHCl_3$).

5.2.9 HF-9: Crinine

Summary formula: $C_{16}H_{17}NO_3$

Based on 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. 22) [22; 141].

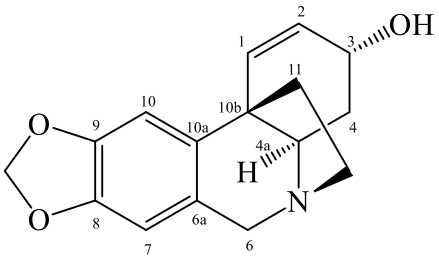


Fig.22. Crinine

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, $CDCl_3$) δ: 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 Hz, *J* = 5.2 Hz, H-2), 5.89 (1H, d, *J* = 17.9 Hz, overlapped, OCH_2O), 5.88 (1H, d, *J* = 17.9 Hz, overlapped, OCH_2O), 4.38 (1H, d, *J* = 16.6 Hz, H-6), 4.34 (1H, ddd, *J* = 6.0 Hz, *J* = 5.2 Hz, *J* = 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 Hz, *J* = 9.0 Hz, *J* = 6.0 Hz, H-12), 2.18 (1H, ddd, *J* = 12.2 Hz, *J* = 9.0 Hz, *J* = 4.3 Hz, H-11), 2.05 (1H, ddd, *J* = 13.9 Hz, *J* = 4.3 Hz, 1.8 Hz, H-4), 1.93 (1H, ddd, *J* = 12.2 Hz, *J* = 10.7 Hz, *J* = 6.0 Hz, H-11), 1.74 (1H, ddd, *J* = 13.9 Hz, *J* = 13.6 Hz, *J* = 6.0 Hz, H-4).

^{13}C NMR (125 MHz, $CDCl_3$) δ: 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 ° (c = 0.26, $CHCl_3$)

5.2.10 HF-10: Lycorine

Summary formula: $C_{16}H_{17}NO_4$

Based on 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 lycorine (Fig. 23) [142].

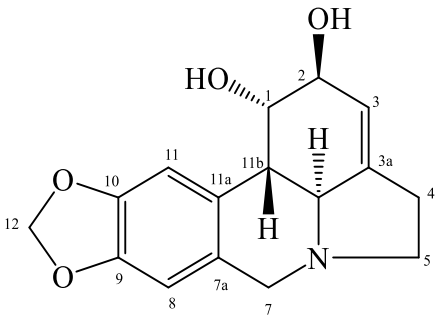


Fig. 23. Lycorine

Molecular weight: 287.31

MS analysis

EIMS *m/z* (%): 287(35), 286(30), 268(20), 250(15), 227(70), 226(100), 211(8), 147(15).

NMR analysis

^{1}H NMR (500 MHz, DMSO) δ: 6.80 (1H, s), 6.67 (1H, s), 5.95–5.93 (2H, m), 5.38–5.35 (1H, m), 4.87(1H, d, *J* = 5.3 Hz), 4.76 (1H, d, *J* = 3.8 Hz), 4.28–4.25 (1H, m), 4.01 (1H, d, *J* = 14.0 Hz), 3.99–3.95 (1H, m), 3.35–3.32 (1H, m, overlapped), 3.32 (1H, d, *J* = 14.0 Hz, overlapped), 3.21–3.16 (1H, m), 2.60 (1H, d, *J* = 10.5 Hz), 2.53–2.37 (1H, m), 2.20 (1H, dd, *J* = 17.3 Hz, *J* = 8.6 Hz).

^{13}C NMR (125 MHz, DMSO) δ: 145.8, 145.4, 141.8, 129.9, 129.8, 118.7, 107.2, 105.2, 100.7, 71.9, 70.4, 61.0, 56.9, 53.5, 40.3, 28.3

Optical rotation

$[\alpha]^{25}_D$ = -104° (c = 0.05, MeOH:$CHCl_3$).

5.2.11 HF-11: 11-Hydroxyvittatine

Summary formula: $C_{16}H_{17}NO_4$

Based on 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 11-hydroxyvittatine (24) [107].

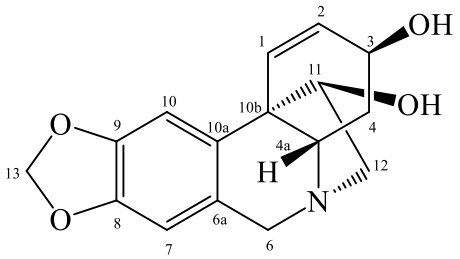


Fig. 24. 11-Hydroxyvittatine

Molecular weight: 287.31

MS analysis

EIMS *m/z* (%): 287(5), 258(100), 211(15), 186(20), 181(23), 153(13), 128(25), 115(25).

NMR analysis

^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.85 (1H, s), 6.48 (1H, s), 6.41 (1H, d, *J* = 10.3 Hz), 6.36 (1H, dd, *J* = 10.3 Hz, *J* = 4.9 Hz), 5.92–5.90 (2H, m), 4.42–4.38 (1H, m), 4.32 (1H, d, *J* = 17.1 Hz), 4.01–3.98(1H, m), 3.69 (1H, d, *J* = 17.1 Hz), 3.43–3.36 (2H, m), 3.26 (1H, dd, *J* = 14.0 Hz, *J* = 2.9 Hz), 2.26 (1H, td, *J* = 14.0 Hz, *J* = 4.4 Hz), 1.95–1.90 (1H, m).

^{13}C NMR (126 MHz, $CDCl_3$) δ: 146.5, 146.3, 135.1, 134.2, 126.9, 126.7, 106.9, 103.2, 100.9, 80.1, 64.2, 63.5, 62.3, 61.4, 50.1, 32.3

Optical rotation

$[\alpha]^{25}_D$ = + 20.0° (c = 0.1, MeOH).

5.2.12 HF-12: Homolycorine

Summary formula: $C_{18}H_{21}NO_4$

Based on the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the homolycorine-type alkaloid homolycorine (Fig. 25) [43].

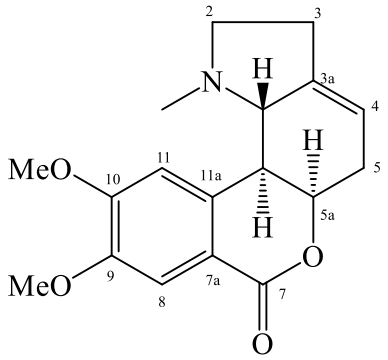


Fig. 25. Homolycorine

Molecular weight: 315.36

MS analysis

EIMS *m/z* (%): 315(<1), 206(<1), 178(2), 109(100),150(1), 108(23), 94(3), 82(3).

NMR analysis

^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.56 (1H, s, H-8), 6.96 (1H, s, H-11), 5.50 (1H, d, *J* = 2.3 Hz, H-3), 4.80 (1H, t, H-5a), 3.95 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 2.71 (1H, m, H-11c), 2.63 (3H, m, H-2, H-11b), 2.50 (2H, m, H-3), 3.12 (1H, ddd, *J* = 9.8 Hz, *J* = 3.3 Hz, *J* = 7.2 Hz, H-2), 2.39 (2H, m, H-5), 2.23 (1H, q, *J* = 9.8 Hz, H-2), 2.00 (3H, s, NCH_3).

^{13}C NMR (125 MHz, $CDCl_3$) δ: 165.9 (C-7), 153.1 (C-10), 148.9 (C-9), 140.9 (C-3a), 137.8 (C-11a), 116.9 (C-7a), 115.2 (C-4), 111.9 (C-8), 110.8 (C-11), 77.7 (C-5a), 66.6 (C-11c), 56.6 (C2), 56.4 (OCH_3), 56.2 (OCH_3), 44.2 (NCH_3), 43.8 (C-11b), 31.3 (C-5), 28.1 (C-3).

Optical rotation

$[\alpha]^{25}_D$ = + 80° (c = 0.02, $CHCl_3$).

5.2.13 HF-13: Pancracine

Summary formula: $C_{16}H_{17}NO_4$

Based on the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the montanine-type alkaloid pancracine (Fig. 26) [143].

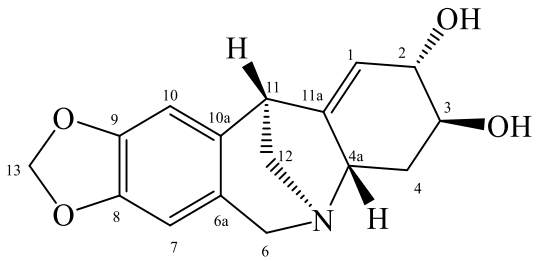


Fig. 26. Pancracine

Molecular weight: 287.31

MS analysis

EIMS *m/z* (%): 287(100), 286(23), 270(20), 243(26), 223(30), 214(25), 199(30), 185(41), 128(20), 115(25).

NMR analysis

^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.61 (1H, s, H-10), 6.54 (1H, s, H-7), 5.88 – 5.85 (2H, m, -OCH_2O-), 5.55 – 5.52 (1H, m, H-1), 4.32 (1H, d, *J* = 16.4 Hz, H-6), 3.97 – 3.94 (1H, m, H-3), 3.88 – 3.85 (1H, m, H-2), 3.83 (1H, d, *J* = 16.4 Hz, H-6), 3.53 – 3.46 (1H, m, H-4a), 3.39 – 3.37 (1H, m, H-11), 3.08 – 3.06 (2H, m, H-12), 2.13 – 2.07 (1H, m, H-4), 1.58 (1H, td, *J* = 12.2 Hz, *J* = 2.7 Hz, H-4).

^{13}C NMR (125 MHz, $CDCl_3$) δ: 153.5 (C-11a), 148.3 (C-8), 147.7 (C-9), 133.5 (C-10a), 125.0 (C-6a), 116.4 (C-1), 108.3 (C-10), 107.8 (C-7), 102.1 (-OCH_2O-), 72.5 (C-3), 70.1 (C-2), 61.4 (C-6), 59.5 (C-4a), 56.2 (C-12), 46.7 (C-11), 31.6 (C-4).

Optical rotation

$[\alpha]^{25}_D$ = - 100 ° (c = 0.1, MeOH).

5.2.14 HF-14: Dihydrolycorine

Summary formula: $C_{16}H_{19}NO_4$

Based on 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 dihydrolycorine (Fig. 27) [16].

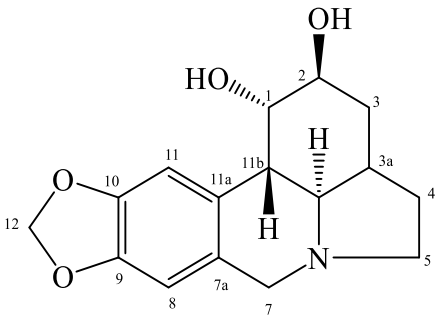


Fig. 27. Dihydrolycorine

Molecular weight: 289.33

MS analysis

EIMS *m/z* (%): 288(100), 272(7), 254(3), 214(6), 200(2), 187(6), 162(6), 147(15).

NMR analysis

^{1}H NMR (500 MHz, CD_3OD) δ: 6.97 (s, 1H), 6.64 (s, 1H), 5.90 (s, 1H), 5.89 (s, 1H), 4.51 (s, 1H), 4.13 (d, *J* = 15.8 Hz, 1H), 3.94 – 3.87 (m, 1H), 3.68 (d, *J* = 15.8 Hz, 1H), 3.27 – 3.18 (m, 1H), 3.04 (dd, *J* = 11.1 Hz, *J* = 5.8 Hz, 1H), 2.86 – 2.78 (m, 1H), 2.58 – 2.48 (m, 2H), 2.18 – 2.04 (m, 2H), 1.96 – 1.82 (m, 2H).

^{13}C NMR (126 MHz, CD_3OD) δ: 147.9, 147.2, 132.3, 129.3, 107.7, 106.2, 102.0, 69.9, 69.7, 59.5, 54.7, 54.1, 38.5, 36.9, 29.8, 28.1.

Optical rotation

$[\alpha]^{25}_D$ = + 99.5° (c = 3.0, 1% H_2SO_4).

5.2.15 HF-15: 9-*O*-Demethylhomolycorine

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

Based on the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the homolycorine-type alkaloid 9-*O*-demethylhomolycorine (Fig. 28) [43].

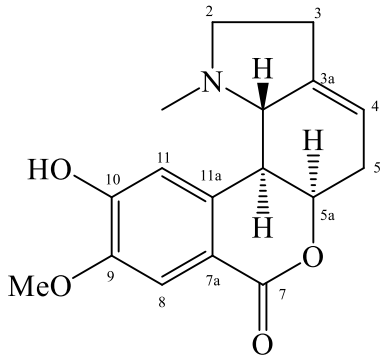


Fig. 28. 9-*O*-Demethylhomolycorine

Molecular weight: 301.34

MS analysis

EIMS *m/z* (%): 164(2), 110(8), 109(100), 108(25), 94(3), 93(2), 82(3), 81(2), 77(1), 65(2).

ESI/MS *m/z* (%): 302(100, M+1), 252(5), 181(3).

NMR analysis

^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.59 (1H, s, H-8), 6.98 (1H, s, H-11), 5.52 – 5.48 (1H, m, H-4), 4.79 – 4.76 (1H, m, H-5a), 3.94 (3H, s, OCH_3), 3.17 – 3.12 (1H, m, H-2), 2.76 – 2.70 (1H, m, H-11c), 2.66 (1H, dd, J = 9.7 Hz, J = 2.1 Hz, H-11b), 2.62 – 2.58 (2H, m, H-5), 2.53 – 2.47 (2H, m, H-3), 2.26 (1H, q, J = 9.5 Hz, H-2), 2.00 (3H, s, NCH_3).

^{13}C NMR (125 MHz, $CDCl_3$) δ: 165.6 (C-7), 150.8 (C-10), 145.6 (C-9), 140.6 (C-3a), 136.7 (C-11a), 117.6 (C-7a), 116.0 (C- 8), 115.4 (C-4), 110.3 (C-11), 77.5 (C-5a), 66.5 (C-11c), 56.5 (C-2), 56.3 (OCH_3), 44.0 (C-11b), 43.7 (NCH_3), 31.2 (C-5), 27.9 (C-3).

Optical rotation

$[\alpha]^{25}_D$ = + 215.7° (c = 0.102, MeOH).

5.2.16 HF-16: Zephyranthine

Summary formula: $C_{16}H_{19}NO_4$

Based on 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 zephyranthine (Fig. 29) [144].

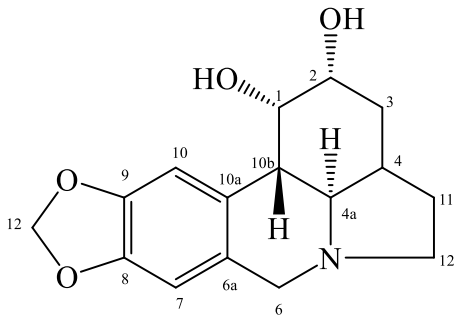


Fig. 29. Zephyranthine

Molecular weight: 289.331

MS analysis

EIMS *m/z* (%): 289 $[M]^+$ (40), 288 $[M-1]^+$ (100), 272(7), 270(7), 254(3), 226(5), 214(8), 187(6), 147(17).

NMR analysis

^{1}H NMR(500 MHz, CD_3OD) δ: 7.00 (s, 1H), 6.71 (s, 1H), 5.93 –5.91 (d, *J* = 1.5, 2H), 4.51 (s, 1H), 4.27 (d, *J* = 15.0, 1H), 3.90 (ddd, *J* = 11.5 Hz, *J* = 5.0 Hz, *J* = 2.5, 1H), 3.81 (d, *J* = 15.0, 1H), 3.37 (td, *J* = 10.0 Hz, *J* = 9.5, 1H), 3.19 (dd, *J* = 11.0 Hz, *J* = 6.5, 1H), 3.02 (ddd, *J* = 10.0 Hz, *J* = 9.5 Hz, *J* = 3.0 Hz, 1H), 2.66 (d, *J* = 11.0 Hz, 1H), 2.58 (qd, *J* = 6.5 Hz, *J* = 1.5 Hz, 1H), 2.11 (ddd, *J* = 13.5 Hz, *J* = 11.5 Hz, *J* = 6.5 Hz, 1H), 2.04 (m, 1H), 1.98 (m, 1H),1.89 (ddd, *J* = 13.5 Hz, *J* = 5.0 Hz, *J* = 1.5 Hz, 1H).

^{13}C NMR(75 MHz, CD_3OD) δ: 148.6, 147.4, 132.6, 127.4, 107.9, 106.5, 102.3, 69.8, 69.4, 61.4, 55.6, 54.1, 38.4, 37.4, 29.2, 28.3

Optical rotation

$[\alpha]^{20}_D$ = -30.6° (c = 0.56; MeOH).

5.2.17 HF-17: 9-*O*-Demethyl-7-*O*-methyllycorenine

Summary formula: $C_{18}H_{23}NO_4$

Based on the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as the homolycorine-type alkaloid 9-*O*-demethyl-7-*O*-methyllycorenine (Fig. 30) [145].

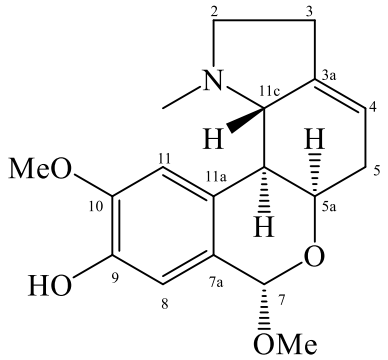


Fig. 30. 9-*O*-Demethyl-7-*O*-methyllycorenine

Molecular weight: 318.17

MS analysis

EIMS *m/z* (%): 286(4), 285(2), 177(8), 110(8), 109(100), 108(16), 94(3), 93(1), 82(2), 81(2).

ESI/MS *m/z* (%): 318 $[M + H]^+$

NMR analysis

^{1}H NMR (500 MHz, CD_3OD) δ: 3.09 (1H, dd, *J* = 9.2 Hz, *J* = 9.2 Hz, H-2*α*), 2.18 (1H, m, H-2*β*), 2.42 (2H, m, H-3), 5.42 (1H, m, H-4), 2.54 (1H, m, H-5*α*), 2.18 (1H, m, H-5*β*),4.14 (1H, dd, *J* = 5.8 Hz, *J* = 1.8 Hz, H-5*α*), 5.34 (1H, s, H-7), 6.64 (1H, s, H-8), 6.84 (1H, s, H-11), 2.33 (1H, dd, *J* = 9.8 Hz, *J* = 1.8 Hz, H-11b), 2.74 (1H, m, H-11c), 3.39 (3H, s, C7-OCH_3), 3.77 (3H, s, C10-OCH_3), 2.02 (3H, s, N-CH_3).

^{13}C NMR (125 MHz, CD_3OD) δ: 57.7 (C-2), 28.7 (C-3), 140.7 (C-3a), 117.7 (C-4), 32.6 (C-5), 68.1 (C-5a), 99.7 (C-7), 130.0 (C- 7a), 113.6 (C-8), 148.8 (C-9), 147.1 (C-10), 115.4 (C-11), 127.5 (C-11a), 44.5 (C-11b), 69.0 (C-11c), 56.5 (C7-OCH_3), 55.6 (C10-OCH_3), 44.1 (N-CH_3).

Optical rotation

$[\alpha]^{25}_D$ = +252 ° (c = 0.10, $CHCl_3$).

5.2.18 HF-18: 9-*O*-Demethyllycorenine (new compound)

Summary formula: $C_{17}H_{21}NO_4$

Based on the performed experiments (MS, NMR, optical rotation) and the comparison of the acquired data with the literature, the compound was identified as a new homolycorine-type alkaloid 9-*O*-demethyllycorenine. (Fig. 31).

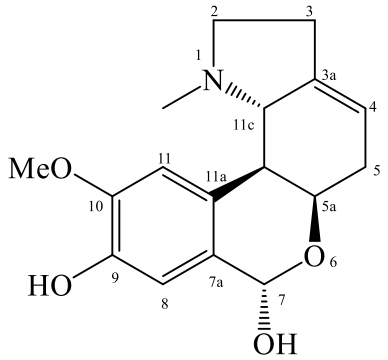


Fig. 31. 9-*O*-Demethyllycorenine

The ESI-HRMS of this compound showed a molecular ion peak $[M+H]^+$ at *m/z* 304.1550, corresponding to the formula $C_{17}H_{21}NO_4^+$ (calc. 304.1543). (Fig. 32).

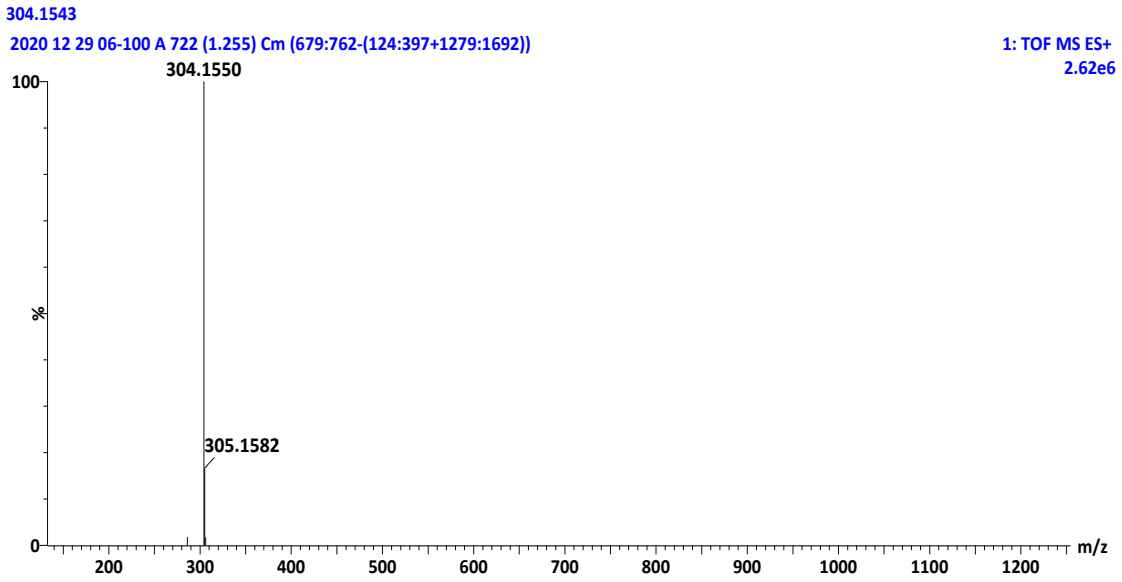


Fig.32. ESI-HRMS spectrum of 9-*O*-demethyllycorenine

The lycorenine-type skeleton was proved in the ^{1}H NMR spectrum by the presence of two singlets of a 1,2,4,5-tetrasubstituted benzene ring (δ_H 6.94, H-8; 6.91, H-11), one deshielded singlet of a benzylic proton (δ_H 6.01, H-7), a multiplet of olefinic protons (δ_H 5.50–5.46, H-4), and so on. Compared to the resonances of three lycorenine methyls, only two signals of the methyl

groups were recognized in the ^{1}H NMR spectrum of this compound - the deshielded *O*-methyl (δ_H 3.89, s) and the methyl of the *N*CH_3 group (δ_H 2.10, s) (Fig. 33).

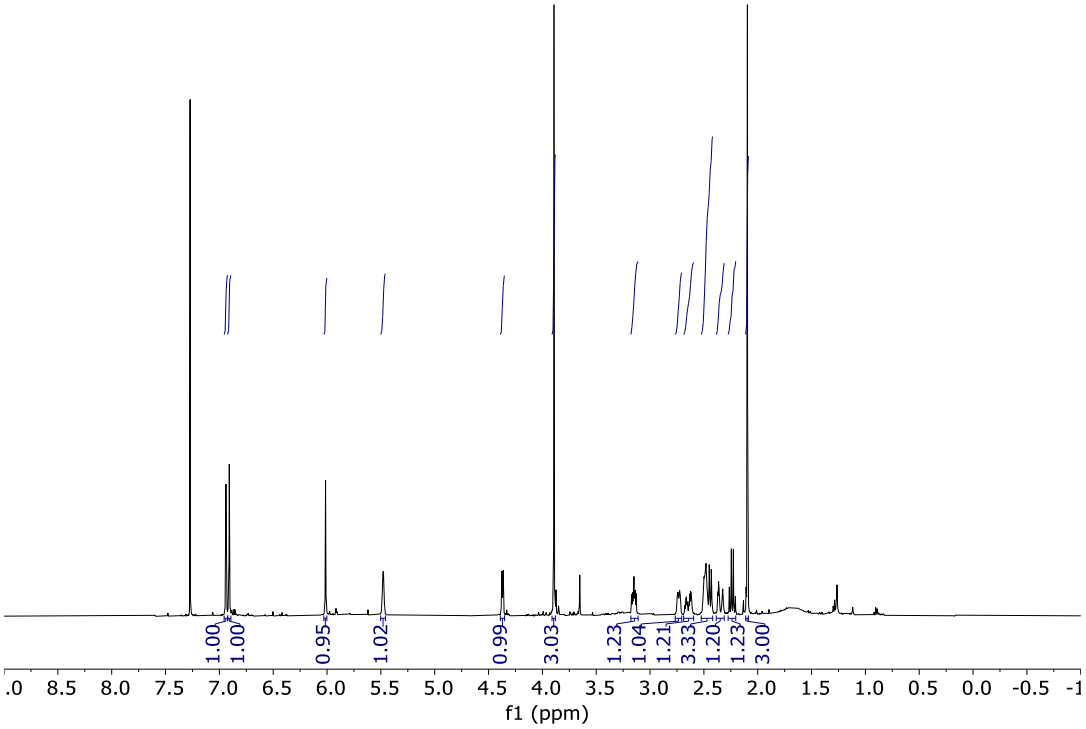


Fig.33. ^{1}H NMR spectrum of 9-*O*-demethyllycorenine in $CDCl_3$

By employing the 2D NMR experiments, the constitution of this compound was confirmed (Fig. 34). The crucial position of the *O*CH_3 was determined by gHMBCAD interactions (Fig. 35) and supported by a cross-peak of its methyl protons and H-11 in the NOESY spectrum (Fig. 36). This experiment was used for the establishment of the relative configuration as follows; the cross-peak of H-5a/H-11b in the NOESY spectrum presented in the *cis* position for these protons, and no through-space interaction of H-5a/ H-7, and H-11b/ H-11c, respectively, determined H-11b/H-11c with a *trans* orientation. Moreover, H-11b coupled to H11c with ^{3}J = 9.5 Hz, which corresponds to a *trans*-pseudo-diaxial position.

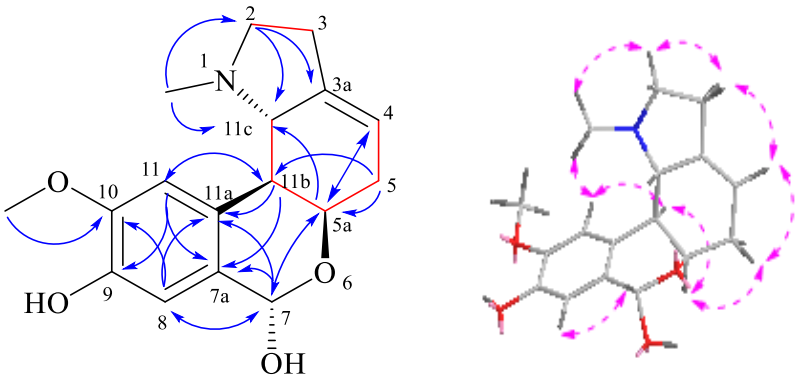


Fig.34. Structural elucidation of 9-*O*-demethyllycorenine showing key gCOSY, gH2BC (Red lines), gHMBCAD (blue arrows), and NOESY (pink dashed arrows) correlations.

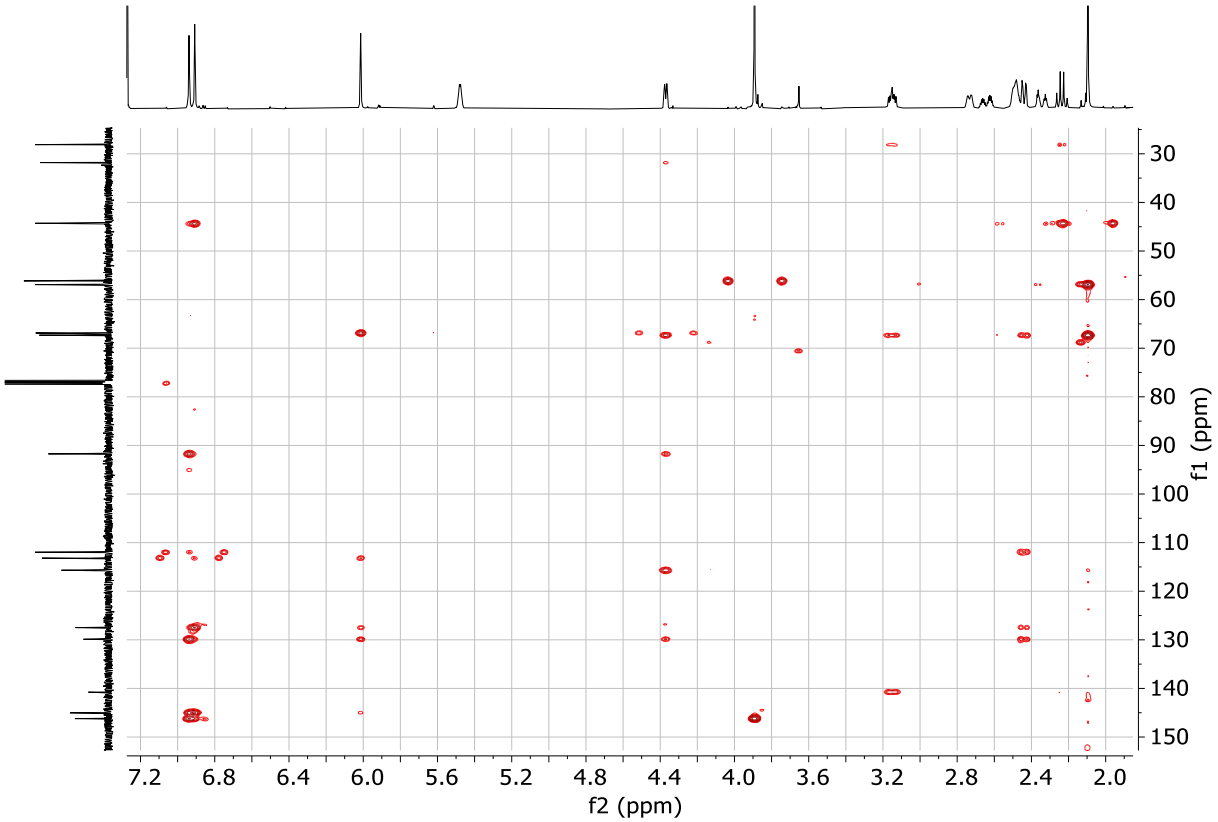


Fig.35. gHMBCAD spectrum of 9-*O*-demethyllycorenine

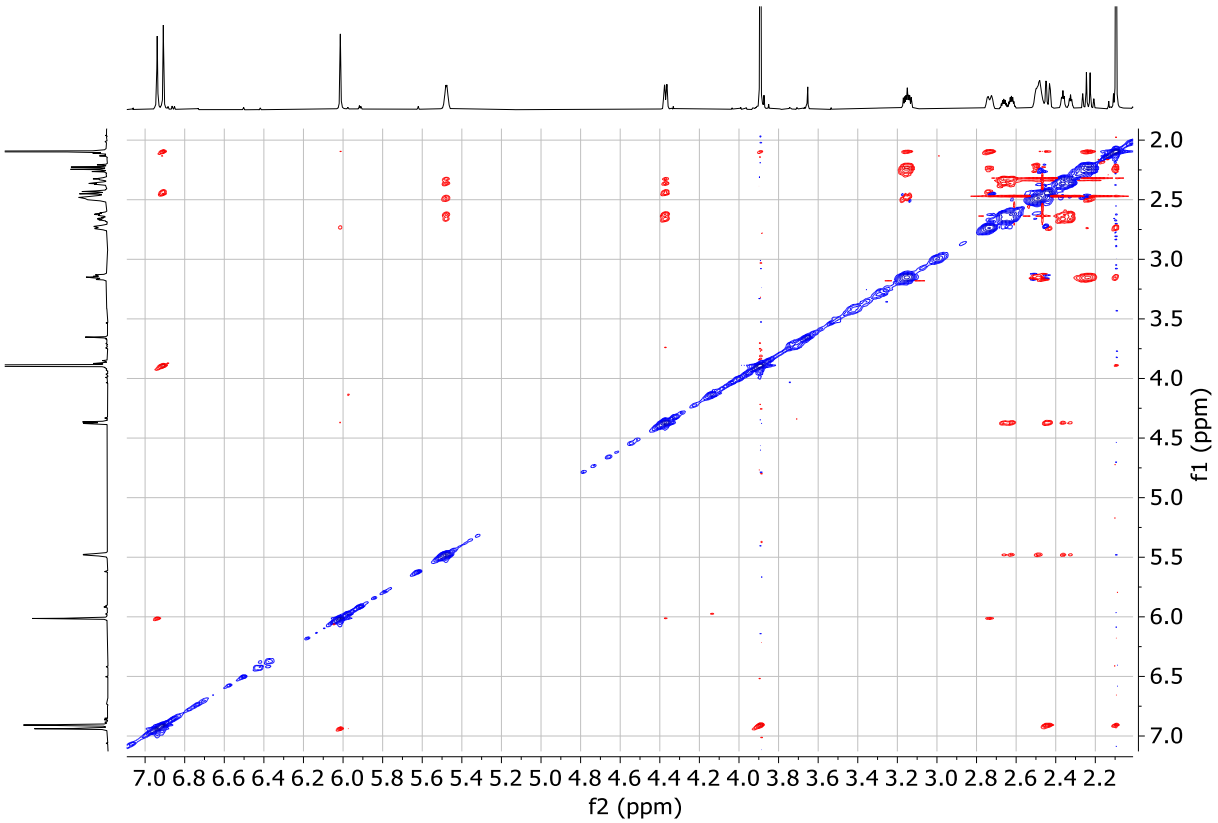
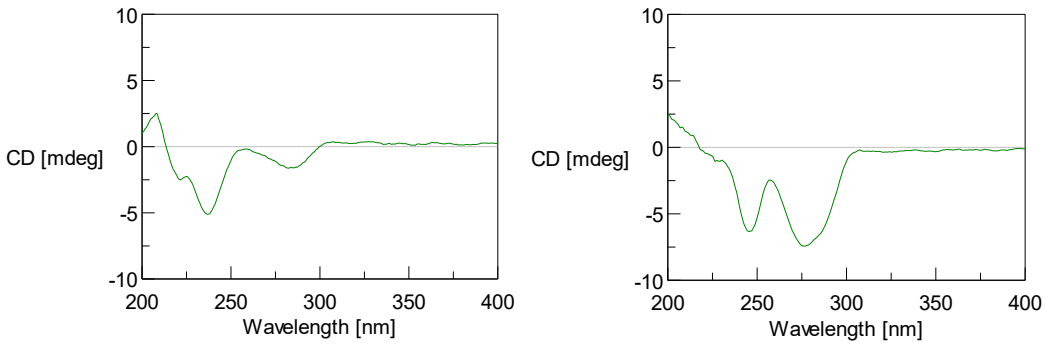


Fig.36. NOESY spectrum of 9-*O*-demethyllycorenine

The absolute configuration was determined as (5aR,7S,11bS,11cS) using chiroptical methods and comparison with published data of similar compounds. The ECD spectrum of 9-O-demethyllycorenine, with negative Cotton effects at 237 nm and 282 nm, was in good agreement with that of lycorenine (Fig.37). The dextrorotatory value is in accordance with those found for other alkaloids possessing a lycorenine skeleton, such as lycorenine, nerinine, and oduline [146].



9-*O*-demethyllycorenine lycorenine

Fig. 37. ECD data of 9-*O*-demethyllycorenine and lycorenine

NMR analysis

^{1}H NMR (500 MHz, CDCl3) δ: 6.94 (s, 1H, H-8), 6.91 (s, 1H, H-11), 6.01 (s, 1H, H-7), 5.50 – 5.46 (1H, m, H-4), 4.37 (1H, dd, *J* = 5.5 Hz, *J* = 1.8 Hz, H-5a), 3.89 (3H, s, 10-OCH_3), 3.15 (1H, ddd, *J* = 9.4 Hz, *J* = 6.7 Hz, *J* = 3.7 Hz, H-2), 2.73 (1H, d, *J* = 9.5 Hz, H-11c), 2.68 – 2.59 (1H, m, H-5), 2.53 – 2.46 (2H, m, H-3), 2.44 (1H, dd, *J* = 9.5 Hz, *J* = 1.8 Hz, H-11b), 2.39 – 2.29 (1H, m, H-5), 2.24 (1H, dt, *J* = 9.4 Hz, *J* = 9.4 Hz, H-2), 2.10 (3H, s, N1-CH_3).

^{13}C NMR (126 MHz, $CDCl_3$) δ: 146.2 (C-10), 145.0 (C-9), 140.8 (C-3a), 129.9 (C-11a), 127.5 (C-7a), 115.7 (C-4), 113.2 (C-8), 112.0 (C-11), 91.8 (C-7), 67.3 (C-11c), 66.9 (C-5a), 56.9 (C-2), 56.2 (10-OCH_3), 44.4 (C-11b), 44.3 (N1-CH_3), 31.8 (C-5), 28.1 (C-3).

Optical rotation

$[\alpha]^{24}_{D}$ = +60 ° (c = 0.10, MeOH).

5.3. Biological activities of the isolated alkaloids from *Hippeastrum* cultivars and *Hippeastrum* x *hybridum* cv. Ferrari

5.3.1 Cytotoxic activity of the isolated alkaloids from *Hippeastrum* cultivars

Three of the isolated alkaloids from *Hippeastrum* cultivars which have not been described by our laboratory previously (montanine, vittatine, and hippeastrine) were screened for their cytotoxic activity on a panel of human cancer cells of different histotype: Jurkat (acute T cell leukemia, MOLT-4 (acute lymphoblastic leukemia), A549 (lung adenocarcinoma), HT-29 (colorectal adenocarcinoma), PANC-1 (pancreatic epithelial carcinoma), A2780 (ovarian cancer), HeLa (cervical adenocarcinoma), MCF-7 (breast adenocarcinoma) and SAOS-2 (osteosarcoma). In parallel, normal MRC-5 human fibroblasts were used to determine the compounds' overall toxicity against non-cancer cells, The cytotoxic activity of these alkaloids was evaluated using the WST-1 metabolic activity assay. To find the best of them with very high cytotoxicity, the alkaloids were tested for growth inhibitory activity in all 10 cell lines at a single dose of 10 μm (Fig. 38). For each alkaloid tested, the sensitivity in an individual cell line and the mean growth percent (GP) value was calculated as an average of 10 cell lines proliferation in percent (Table.10). The threshold GP value for this screen was < 50% (50% tumor growth inhibition), indicating good activity at 10 μM.

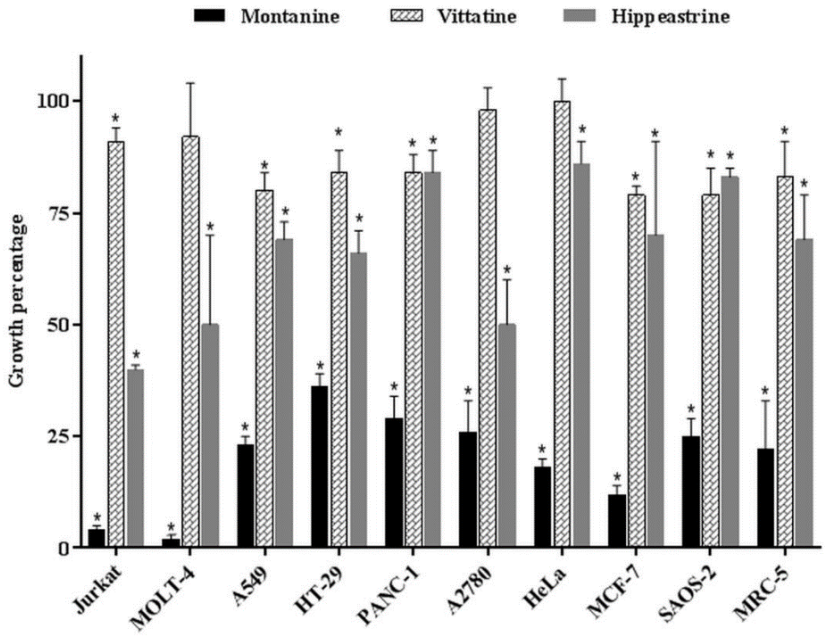


Fig. 38. The growth inhibitory effect of montanine, vittatine and hippeastrine following a single-dose exposure at a concentration of 10 μM on 9 cancer cell lines (Jurkat, MOLT-4, A549, HT-29, PANC-1, A2780, HeLa, MCF-7, SAOS-2) and the non-cancer cell line MRC-5 using WST-1 cytotoxicity assay. Bars indicate mean ± SD, n = 3. * - Significantly different from 0.1% DMSO mock treated control ($p \leq 0.05$).

Table 10. Cytotoxicity of montanine, vittatine, and hippeastrine following a single-dose exposure at a concentration of 10 μM. Doxorubicin at 1 μM was used as a reference drug. Data are shown as mean values ± SD of at least three independent experiments and are expressed as a percent of the proliferation of 0.1% DMSO mock treated control cells (100 %).

Cell type	Montanine	Vittatine	Hippeastrine	Doxorubicin
Jurkat	4 ± 1	91 ± 3	40 ± 1	2 ± 0
MOLT-4	2 ± 1	92 ± 12	50 ± 20	0 ± 0
A549	23 ± 2	80 ± 4	69 ± 4	11 ± 5
HT-29	36 ± 3	84 ± 5	66 ± 5	47 ± 4
PANC-1	29 ± 5	84 ± 4	84 ± 5	78 ± 3
A2780	26 ± 7	98 ± 5	50 ± 10	5 ± 1
HeLa	18 ± 2	100 ± 5	86 ± 5	11 ± 6
MCF-7	12 ± 2	79 ± 2	70 ± 21	37 ± 3
SAOS-2	25 ± 4	79 ± 6	83 ± 2	17 ± 5
MRC-5	22 ± 11	83 ± 8	69 ± 10	29 ± 3

The most active alkaloid in the field of cytotoxicity and antiproliferative activity seemed to be montanine which was found to inhibit Jurkat, MOLT-4, A549, HT-29, PANC-1, A2780, HeLa, MCF-7, and SAOS-2 cancer cell growth with a score ≤ 50% at 10 μM concentration (Table. 11).

Table 11. Sensitivity to the antiproliferative activities of montanine, vittatine, and hippeastrine following a single-dose exposure at a concentration of 10 μM. Doxorubicin at 1 μM was used as a reference drug.

Compound	Mean GP[a]	Range of GP[b]	Most sensitive cell lines	% inhibition
Montanine	20	2 - 36	MOLT-4, Jurkat, MCF-7	2, 4, 12
Vittatine	87	79 - 100	MCF-7, SAOS-2, A549	79, 79, 80
Hippeastrine	67	40 - 86	Jurkat, MOLT-4, A2780	40, 50, 50
Doxorubicin	24	0 - 78	MOLT-4, Jurkat, A2780	0, 2, 5

[a]Mean growth percent (GP) value was calculated for each compound as an average of 9 cell lines proliferation in percent. [b]Range of growth percentage, as well as the three most sensitive cell lines with growth percentage values, are indicated for each compound.

Thus, IC_{50} values of montanine in the range below 10 μM were determined as shown in Table.12. Montanine showed the highest activity towards Jurkat, MOLT-4, and A549 cells. Jurkat cells were highly sensitive to montanine treatment, with an IC_{50} of 1.04 μM [17].

Table 12. IC_{50} values of montanine for human cancer and non-cancer cells [a, b]. Data are shown as mean values ± SD of at least three independent experiments and are expressed as a percent of the proliferation of 0.1% DMSO mock treated control cells (100 %).

Cell type	Montanine
Jurkat	1.04 ± 0.14
MOLT-4	1.26 ± 0.11
A549	1.09 ± 0.31
HT-29	1.35 ± 0.47
PANC-1	2.30 ± 0.45
A2780	1.67 ± 0.29
HeLa	1.99 ± 0.22
MCF-7	1.39 ± 0.21
SAOS-2	1.36 ± 0.49
MRC-5	1.79 ± 0.50

[a] Results are expressed in μM. [b] Results are the mean values ± standard deviations of at least three independent replications

5.3.2 Cytotoxic activity of the isolated alkaloids from *Hippeastrum* x *hybridum* cv. Ferrari

All of the isolated alkaloids from *Hippeastrum* x *hybridum* cv. Ferrari has been screened for their cytotoxic activity on a panel of human cancer cells of different histotypes: Jurkat, MOLT-4, A549, HT-29, PANC-1, A2780, HeLa, MCF-7, and SAOS-2. To determine overall toxicity against non-cancer cells, normal MRC-5 human fibroblasts were used. To find the best of them with very high cytotoxicity, the alkaloids were tested for growth inhibitory activity in all 10 cell lines at a single dose of 10 μm using the WST-1 metabolic activity assay, the most active alkaloids were labeled by red color. (Table. 13).

Table 13. Cytotoxic activity of alkaloidal extract of *Hippestrum* x *hybridum* cv. Ferrari and isolated Amaryllidaceae alkaloids following a single dose exposure at a concentration of 10 μM. Doxorubicin at 1μM was used as a reference drug.

	Cell type									
Compound	**Jurkat**	**MOLT-4**	**A549**	**HT-29**	**PANC1**	**A2780**	**HeLa**	**MCF-7**	**SAOS-2**	**MRC-5**
Hippestrum x *hybridum* cv. Ferrari	8	3	28	37	39	39	24	13	31	26
Dihydro-2-*O*-acetyllycorine (HF-1)	96	82	86	82	94	105	98	83	71	82
Montanine (HF-2)	4	2	23	36	29	26	18	12	25	22
Haemanthamine (HF-3)	18	6	33	5	37	38	16	17	34	32
Tazettine (HF-4)	93	95	83	57	76	101	84	94	92	-
1, 2-*O*,*O'*-Diacetyl-dihydrolycorine (HF-5)	91	75	100	91	117	100	97	94	93	82
Vittatine (HF-8)	91	92	80	84	84	98	100	79	79	83
Crinine (HF-9)	113	109	105	98	102	85	104	112	98	-
Lycorine (HF-10)	5	9	27	5	36	39	15	27	27	25
Homolycorine (HF-12)	78	110	114	199	100	96	108	102	97	97
Pancracine (HF-13)	17	1	29	39	52	40	28	18	26	39
9-*O*-Demethylhomolycorin (HF-15)	100	100	109	106	92	94	96	102	103	93
Zephyranthine (HF-16)	100	90	97	87	98	101	102	95	98	96
9-*O*-Demethyllycorenine (HF-18)	96	103	81	88	108	102	97	101	90	99
Doxorubicin	2	0	11	47	78	5	11	37	17	29
DMSO (0,1%)	113	115	106	87	95	98	89	99	102	-

0 - 25	26 - 50	51 - 75	76 - 100

5.3.3 The biological activity of the isolated alkaloids in relation to Alzheimer's disease

Alkaloids isolated from *Hippeastrum x hybridum* cv. Ferrari were tested for their ability to inhibit several enzymes associated with possible therapy for Alzheimer's disease, such as cholinesterases (*h*AChE, *h*BuChE) and prolyloligopeptidase (POP). The values of the inhibitory activity are summarized in Table 14. Interesting values are labeled in bold.

Table 14. The biological activity of the isolated alkaloids in relation to Alzheimer's disease

Compound	***h*AChE IC_{50} (μM) [a]**	***h*BuChE IC50 (μM) [a]**	**POP IC_{50} (μM) [a]**
Hippestrum x *hybridum* cv. Ferrari	51.8 ± 6.1	96.65 ± 10.04	-
Dihydro-2-*O*-acetyllycorine (HF-1)	n.d.	n.d.	344 ± 18
Montanine (HF-2)	>100	>100	>500
Haemanthamine (HF-3)	>500	>500	>500
Tazettine (HF-4)	>500	>500	>500
1, 2-*O*,*O*'-Diacetyl-dihydrolycorine (HF-5)	>100	**68 ± 3**	n.m.
Ismine (HF-6)	82.40 ± 4.39	170.51 ± 7.45	>1000
3-Epimacronine (HF-7)	87.69 ± 3.11	>100	>1000
Vittatine (HF-8)	>100	>100	>500
Crinine (HF-9)	>500	>500	>500
Lycorine (HF-10)	>500	>500	>500
11-Hydroxyvittatine (HF-11)	>100	>100	>500
Homolycorine (HF-12)	64 ± 4	151 ± 20	**173 ± 41**
Pancracine (HF-13)	>500	>500	>500
Dihydrolycorine (HF-14)	>1000	n.m.	n.m.
9-*O*-Demethylhomolycorine (HF-15)	>500	>500	>500
Zephyranthine (HF-16)	>100	>100	**142 ± 10**
9-*O*-Demethyl-7-*O*-methyllycorenine (HF-17)	n.d.	n.d.	n.d.
9-*O*-Demethyllycorenine (HF-18)	>100	>100	n.m.
Galanthamine[c]	1.7 ± 0.1	42 ± 1	>200
Berberine[c]	0.7 ± 0.1	30.7 ± 3.5	142 ± 21
Z-Pro-prolinal[c]	n.d.	n.d.	2.75×10^{-3}

[a] Compound concentration required to decrease enzyme activity by 50%; the values are the mean ± SEM of three independent measurements, each performed in triplicate; [b] tested at 50 μM compound concentration; n.d. stand for not determined; n.m. stand for not measured due to limited amount of isolated alkaloid.

6 DISCUSSION

The plant family Amaryllidaceae is known for its structurally unique isoquinoline alkaloids called Amaryllidaceae alkaloids. Traditionally, plants of this family have been used as folk herbal remedies against various diseases by indigenous people around the world. [47].

The research of these compounds is focusing on the search for substances useful in anticancer therapy, mainly due to their highly selective effect on tumor cells and also the relatively easy isolation of some alkaloids from natural material. The research is also focusing on their ability to inhibit human cholinesterases, prolyl oligopeptides, and glycogen synthase kinase-3β as potential drugs for the treatment of neurodegenerative diseases like Alzheimer´s disease. The most known AAs is galanthamine, which is a long-acting, selective, reversible, and competitive inhibitor of the acetylcholinesterase enzyme, which has been already approved by the FDA for the treatment of mild to moderate stages of Alzheimer's disease, under the name Razadyne© [26].

The plants of the genus *Hippeastrum* received great attention both in the analytical and isolation field. A large number of alkaloids of various structural types have been identified and isolated from milligram to gram amounts from this genus [17; 109]. *Hippeastrum* x *hybridum* cv. Ferrari was selected for a detailed phytochemical and biological study based on GC/MS screening of six *Hippeastrum* horticultural cultivars. GC/MS analysis of these cultivars showed the presence of a wide range of AAs of various structural types, some of which could be identified by mass spectrometry, some of which determined the structural type of Amaryllidaceae alkaloid, others of which could not be identified at all [17]. Six ornamental varieties of *Hippeastrum* cultivars have been screened for their alkaloid profiling by using GC/MS, this spectrometric techniques permitted the detection of 20 compounds, 18 of which were identified as alkaloids based on their MS data and retention indexes. They belong to the crinine, haemanthamine, galanthamine, homolycorine, lycorine, montanine, and tazettine structural types of AAs. Several alkaloids, marked as A1, A2, and A3 were left unidentified due to their low concentrations (< 5% of TIC) and undescribed mass spectra in the literature. The GC/MS data for alkaloid A2 indicated the presence of an intense peak at m/z 109 such as in masonine and homolycorine [132], suggested that the alkaloid belongs to the homolycorine structural-type of AAs. The proportion of individual components of the alkaloid fraction were expressed as a percentage of the total ion current (TIC).

The alkaloid profile of all studied cultivars was interesting and exhibit a higher diversity of different biologically active AAs. The profile was characterized by five major alkaloids.

Lycorine was the most predominant alkaloid identified in all the studied cultivars, the highest concentration was found in *Hippeastrum* cv. Daphne (56% of TIC), in contrast with *Hippeastrum* x *hybridum* cv. Ferrari showed the least concentration of lycorine (9% of TIC). 11-hydroxyvittatine has been found as a major alkaloid in *Hippeastrum* x *hybridum* cv. Ferrari with a concentration of 49%. On the other hand, the GC/MS analysis of the alkaloidal extract of *Hippeastrum* cv. Spartacus revealed the presence of tazettine as an abundant alkaloid with a concentration of 43%. The homolycorine alkaloid has been found as a major alkaloid in *Hippeastrum* cv. Pretty Nymph with a concentration of 40%. The alkaloid profile of *Hippeastrum* cv. Daphne indicated the main constituent which was stated for hippeastrine alkaloid by a concentration of 34%. Other compounds were identified as minor alkaloids including ismine, trisphaeridine, galanthamine, lycoramine, vittatine, 9-*O*-demethyllycosinine B, 11,12-dehydroanhydrolycorine, A2 homolycorine type, montanine, haemanthamine, pancracine, 3-epimacronine, and pseudolycorine.

Additionally, five AAs have been isolated using preparative TLC from studied *Hippeastrum* cultivars (*Hippestrum* cv. Pretty Nymph, *Hippeastrum* cv. Double King, *Hippeastrum* x *hybridum* cv. Ferrari, *Hippeastrum* cv. Artic Nymph, *Hippeastrum* cv. Daphne) The compounds were identified by comparing their mass spectral fragmentation with those of standard reference spectra of alkaloids isolated by our department, and by using 1D, and 2D NMR techniques. These compounds were identified as montanine, vittatine, 11-hydroxyvittatine, lycorine, and hippeastrine respectively.

The alkaloids which have been isolated from *Hippeastrum* cultivars and not described by our laboratory previously, montanine, vittatine, and hippeastrine, were screened for their cytotoxic activity against a panel of human cancer cells, which spanned cell lines from different tissue types (Jurkat, MOLT-4, A549, HT-29, PANC-1, A2780, HeLa, MCF-7 and SAOS-2). In parallel, normal MRC-5 human fibroblasts were employed to determine the overall toxicity against non-cancer cells. The cytotoxic activity of these alkaloids was evaluated using the WST-1 metabolic activity assay. In this screening process, the alkaloids were tested for growth-inhibitory activity in all 10 cell lines at a single dose of 10 μM. For each alkaloid tested, the sensitivity in an individual cell line and the mean growth percent (GP) value were calculated as an average of 10 cell lines proliferation in percent. The threshold GP value for this screen was < 50% (50% tumor growth inhibition), indicating good activity at 10 μM. In this work, the alkaloid montanine was found to strongly decrease the growth of 7 different adherent cancer cell lines of several histotypes by treatment with a single 10 μM dose. Montanine was also able to inhibit the cell growth of human leukemic cell lines Jurkat and MOLT-4 with a single dose

of 10 μM, causing growth percentage GP values of 4% and 2% respectively. Thus, IC_{50} values of montanine in the range below 10 μM were determined. Montanine showed the highest activity towards Jurkat, MOLT-4, and A549 cells. Jurkat cells were highly sensitive to montanine treatment, with an IC_{50} of 1.04 μM.

Structurally, naturally occurring montanine-type alkaloids, montanine, pancracine, coccinine, and manthine are characterized by a common bridged pentacyclic 5,11-methanomorphantridine ring system and are distinguished by positional and stereochemical diversity of oxygen-containing substituents at C-2 and C-3 [143]. These compounds have been approved for their anticancer activity and seem to be promising compounds. Montanine and coccinine were evaluated for their potential anticancer activity against a panel of six human cancer cell lines including several breasts (MCF7, Hs578T, MDA-MB-231), colon (HCT-15), lung (A549), and melanoma (SK-MEL-28). Coccinine and montanine were found to significantly affect the cell viability of almost all the tested cancer cell lines after 48 h of treatment at low micromolar concentrations [147]. Further studies have been approved that the strong effect of montanine and manthine to inhibit the growth of three cancer cell lines resistant to apoptosis (A549, SKMEL-29, U373) and three cancer cell lines sensitive to apoptosis (MCF7, Hs683, B16F10) with IC_{50} values between 5 and 31 μM [148; 149].

However, since previous studies demonstrated that montanine, manthine, and coccinine could effectively suppress the viability and proliferation of human cancer cells, the molecular mechanism of their cytotoxic activity has not yet been fully explored and is still waiting to be described.

The alkaloid extract of selected horticultural cultivar for detailed phytochemical study *Hippeastrum* x *hybridum* cv. Ferrari was also studied for its *h*AChE and *h*BuChE inhibitory activity (IC_{50}, AChE = 51.8 ± 6.1 μM and IC_{50}, BuChE = 96.65 ± 10.04 μM). The obtained values of IC_{50} of this extract were relatively uninteresting and were not decisive for the selection of the plant. On the other hand, GC/MS study showed the presence of more than 13 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. Most of the identified alkaloids were not isolated in our laboratory previously, thus we decided to select this cultivar for study.

Extensive chromatographic purification of the concentrated alkaloidal extract prepared from 25 kg of fresh bulbs of *Hippestrum* x *hybridum* cv. Ferrari led to the isolation of one new and seventeen 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: monanine (montanine HF-2, pancracine HF-13), haemanthamine (haemanthamine HF-3, vittatine HF-8, 11-hydroxyvittatine HF-11), tazettine (tazettine HF-4, 3-epimacronine HF-7), lycorine (diastomers of dihydro-2-*O*-acetyllycorine HF-1, 1,2-*O,O'*-diacetyl-dihydrolycorine HF-5, lycorine HF-10, dihydrolycorine HF-14, zephyranthine HF-16), homolycorine (homolycorine HF-12, 9-*O*-demethylhomolycorine HF-15, 9-*O*-demethyl-7-*O*-methyllycorenine HF-17, 9-*O*-demethyllycorenine HF-18) pancratistatin (ismine HF-6) and crinine (crinine HF-9).

From the above-isolated alkaloids, the major alkaloids were 11-hydroxyvittatine, vittatine, lycorine, pancracine, montanine, homolycorine, tazettine, and haemanthamine, while the other compounds were considered as minor alkaloids. Some of these compounds have been already isolated from the other *Hippestrum* species such as 11-hydroxyvittatine, which has been isolated from *H. vittatum*, *H. aulicum*, *H. solandriflorum*, and *H. papilio* [93; 97; 101; 102; 107]. The alkaloids montanine, lycorine, and vittatine have been isolated from *H. vittatum* [87-89; 93; 100; 101; 104]. The other alkaloids like tazettine, 3-epimacronine, ismine, and haemanthamine were previously isolated from *H.morelianum* [101; 105].

All of the isolated compounds obtained in sufficient amounts were screened for various biological activities related to AD, including *in vitro* AChE, BuChE, POP inhibition potency. The inhibitory activities against *h*AChE and *h*BuChE of the isolated alkaloids and their potential use in therapy of AD are subjects of a long-term study at the Department of Pharmaceutical Botany. In the *h*AChE/*h*BuChE assay, except for 1,2-*O,O´*-diacetyl-dihydrolycorine which demonstrated a mild *h*BuChE inhibition potency with the value of IC_{50} = 68 ± 3 μM, the isolated alkaloids were considered as inactive (IC_{50} > 100 μM). On the other hand, the most active alkaloid in the POP assay was lycorine-type alkaloid zephyranthine which displayed the same POP inhibition potency (IC_{50} = 142 ± 10 μM) as berberine (IC_{50} = 142 ± 21 μM), which is recognized as a POP inhibitor. Homolycorine showed promising POP inhibition activity with a value of IC_{50} = 173 ± 41 μM. In recent studies, POP inhibitors have demonstrated a high potential to become effective antidementia drugs [130]. POP inhibition can represent an additional supporting approach in AD treatment, and hence the POP inhibitory ability of the isolated compounds was tested.

The second reason for choosing *Hippeastrum* x *hybridum* cv. Ferrari was based on the high content of haemanthamine-type alkaloid vittatine. The isolated amount (2.1 g) and the structure of vittatine (HF-8) allowed us, as a continuation of the phytochemical study, to develop new chemical entities by substitution of its free hydroxyl group in position C-3 to inspect the inhibition potency and structure-activity relationship of the novel analogs against enzymes

connected with the potential treatment of AD. The preparation of vittatine-derivatives (8a-8o) has been inspired by our previous work [108], where selected semisynthetic derivatives of haemanthamine displayed promising inhibition potential against cholinesterases. Vittatine (HF-8) belongs to the same structural type as haemanthamine; they both display the same orientation of the 5,10*b*-ethano bridge, and the two alkaloids differ first in substitutions on C-3 where there is a hydroxy group in vittatine instead of the methoxy group in haemanthamine, and secondly in position C-11; there is the only hydrogen in vittatine, but hydroxy in haemnanthamine.

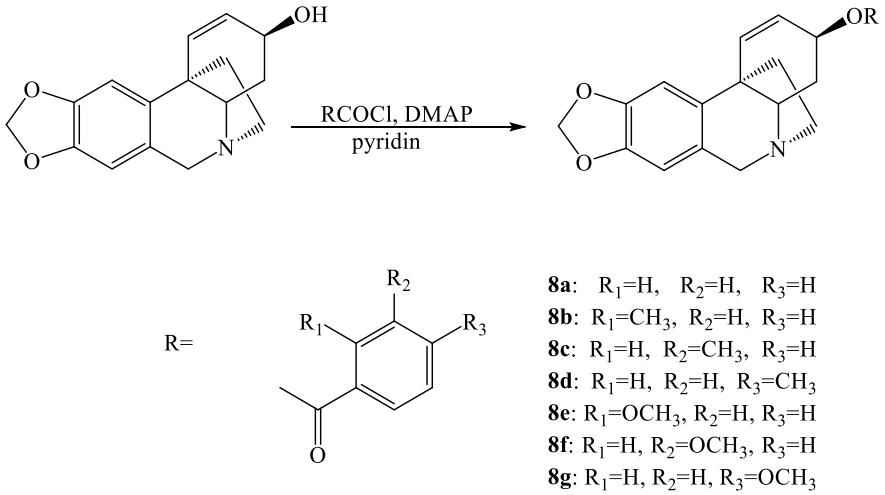


Fig. 39. Synthetic procedure for 8a – 8o from vittatine (HF-8)

The inhibitory activities of the target compounds (8a-8o; Fig. 39) against *h*AChE and *h*BuChE were determined by Ellman´s method using galanthamine and eserine as the reference compounds [128]. The *h*AChE/*h*BuChE inhibitory activity of the derivatives was first screened at a concentration of 100 μM. Compounds displaying inhibition ability >50% against one or both cholinesterases at the tested concentration were selected for the determination of their IC_{50} values. Similar to our previous report on haemanthamine and its analogs [108], the presence of an aromatic acyl moiety was crucial to exert *h*AChE and *h*BuChE inhibitory potency. Within this study, only two vittatine derivatives were potent *h*AChE inhibitors, namely 8h, and 8j, which revealed rather moderate inhibition, with IC_{50} values of 60 ± 2 μM, and 12 ± 1 μM, respectively. On the other hand, three aromatic derivatives (8h, 8m, and 8b) with different

substitutions on the attached aromatic ring exerted strong inhibitory potency against *h*BuChE (IC_{50} < 10 μM). The strongest *h*BuChE inhibition capability was demonstrated by the 3-*O*-(2-nitrobenzoyl)vittatine (8h), 3-*O*-(2-chlorobenzoyl)vittatine (8m), and 3-*O*-(2-methylbenzoylvittatine (8b) with IC_{50} values of 1.4 ± 0.1 μM, 5.4 ± 0.1 μM, and 8.0 ± 0.1 μM respectively. Moreover, the results for 8h, 8m, and 8b showed a selective inhibition pattern for *h*BuChE. Comparing the suitability of substitution and position in the aromatic ester sub-group, the highest inhibition was related to *ortho* substitution in the attached aromatic ring, as in 8h, 8m, and 8b compared with positions *meta* and *para*. The presence of a nitro group in the *ortho* position on the aromatic ring (8h) showed the most pronounced *h*BuChE inhibition ability. The presence of substitution in either the *meta* or *para* position was connected with a dramatic decrease in *h*BuChE inhibition activity. To explain these findings in more detail, further docking studies for a wider range of haemanthamine and vittatine derivatives will be undertaken soon.

Since the synthesis of semisynthetic derivatives of AAs was not the content of this dissertation, but only a continuation, the remaining results of the whole study can be found in the work of Al Shammari et.al. [150].

In conclusion, we can state that *Hippeastrum* x *hybridum* cv. Ferrari is a rich source of diverse Amaryllidaceae alkaloids, especially of the haemanthamine-type, and montanine with important biological activities and it is a promising genus for further phytochemical research.

7 ABSTRACT

Charles University in Prague, Faculty of Pharmacy in Hradec Králové

Department of Pharmaceutical Botany

Candidate: **MSc. Latifah Al Shammari**

Supervisor: **Prof. Ing. Lucie Cahlíková, Ph.D.**

Title of Doctoral Thesis: **Alkaloids of the genus *Hippeastrum* (Amaryllidaceae): isolation, identification, biological activity.**

Hippeastrum x *hybridum* cv. Ferrari was chosen based on results of previous screening studies for detailed phytochemical study for the purpose of isolation of the widest range of AAs. From 25 kg of fresh bulbs was obtained 29,46 g of purified alkaloidal extract, which was processed using column chromatography. Finally, 18 pure alkaloids were isolated including one new homolycorine-type alkaloid (9-*O*-demethyllycorenine). All compounds were identified by spectrometric techniques (GC-MS, ESI-MS, NMR, optical rotation) and by comparison with literature data.

All alkaloids, isolated in sufficient amount, were tested for their biological activities associated with Alzheimer's disease (inhibition of *h*AChE, *h*BuChE, and POP), and cytotoxic activity. The inhibitory activity against human cholinesterases was determined *in vitro* by a modified Ellman's spectrophotometric method. In the *h*AChE/*h*BuChE assay, except for 1,2-*O,O´*-diacetyl-dihydrolycorine which demonstrated a mild *h*BuChE inhibition potency with IC_{50} values of 68 ± 3 μM, the isolated alkaloids were considered as inactive (IC_{50} > 100 μM). POP inhibition activity was determined by spectrophotometric method, the most active alkaloids in the POP assay were zephyranthine (IC_{50} = 142 ± 10 μM) and homolycorine (IC_{50} = 173 ± 41 μM). These compounds displayed activity comparable to used standard berberine (IC_{50} = 142 ± 21 μM).

The majority of isolated alkaloids were screened for their cytotoxicity on a panel of human cancer cells of different histotypes (Jurkat, MOLT-4, A549, HT-29, PANC-1, A2780, HeLa, MCF-7 a SAOS-2). Among tested alkaloids, montanine has been considered as the most potent compound to inhibit Jurkat, MOLT-4, A549, HT-29, PANC-1, A2780, HeLa, MCF-7, and SAOS-2 cancer cells. Jurkat cells were the most sensitive cell line to montanine, with the value of IC_{50} of 1.04 ± 0.14 μM.

As a part of the continuation of this study, a pilot series of semisynthetic derivatives of haemanthamine-type alkaloid vittatine were developed. Vittatine derivatives were studied for their *h*AChE/*h*BuChE inhibition potential.

Keywords: *Hippeastrum* x *hybridum* cv. Ferrari, Amaryllidaceae, alkaloids, biological activity, acetylcholinesterase, butyrylcholinesterase, prolyl oligopeptidase, cytotoxicity.

8 ABSTRAKT

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Název práce: **Alkaloidy rodu *Hippeastrum* (Amaryllidaceae): izolace, identifikace, biologická aktivita**

Pěstovaná odrůda *Hippeastrum* x *hybridum* cv. Ferrari byla vybrána pro podrobnou fytochemickou studii za účelem izolace alkaloidů v čistém stavu na základě výsledků screeningových studií. Z 25 kg čerstvých cibulí bylo získáno 29,46 g vyčištěného alkaloidního extraktu, který byl zpracován pomocí sloupcové chromatografie. Bylo izolováno 18 čistých alkaloidů, včetně jednoho nového alkaloidu, který patří do homolykorinového strukturního typu (9-*O*-demethyllykorenin). Všechny izolované alkaloidy byly identifikovány pomocí spektroskopických technik (GC-MS, ESI-MS, NMR, optická rotace) a porovnáním s údaji v literatuře.

Všechny alkaloidy izolované v dostatečném množství byly testovány na biologické aktivity spojené s Alzheimerovou chorobou (inhibice *h*AChE, *h*BuChE a POP) a cytotoxickou aktivitu. Inhibiční aktivita proti lidským cholinesterázám byla stanovena *in vitro* modifikovanou Ellmanovou spektrofotometrickou metodou. V testu *h*AChE / *h*BuChE, s výjimkou 1,2-*O,O*-diacetyl-dihydrolykorinu, který vykazoval mírnou účinnost inhibice *h*BuChE s hodnotami IC_{50} 68 ± 3 μM, byly izolované alkaloidy považovány za neaktivní (IC_{50} > 100 μM). Inhibiční aktivita vůči POP byla stanovena spektrofotometrickou metodou, nejaktivnějšími alkaloidy v POP studii byly zephyranthin (IC_{50} = 142 ± 10 μM) a homolykorin (IC_{50} = 173 ± 41 μM). Tyto sloučeniny vykazovaly aktivitu srovnatelnou s použitým standardem berberinem (IC_{50} = 142 ± 21 μM).

Většina izolovaných alkaloidů byla testována na jejich cytotoxicitu na panelu lidských rakovinných buněk odlišného histotypu (Jurkat, MOLT-4, A549, HT-29, PANC-1, A2780, HeLa, MCF-7 a SAOS-2). Z testovaných alkaloidů byl montanin identifikován jako látky s nejvyšším cytotoxickým potenciálem vůči Jurkat, MOLT-4, A549, HT-29, PANC-1, A2780, HeLa, MCF-7 a SAOS-2.

V rámci pokračování této studie byla připravena pilotní řada polosyntetických derivátů alkaloidu haemanthaminového typu vittatinu. U těchto derivátů byl studován jejich inhibiční potenciál *h*AChE / *h*BuChE.

Klíčová slova: *Hippeastrum* x *hybridum* cv. Ferrari, Amaryllidaceae, alkaloidy, biologická aktivita, acetylcholinesteráza, butyrylcholinesteráza, prolyl oligopeptidáza, cytotoxicita.

9 COMPENDIUM OF PUBLICATIONS

9.1 Publications related to dissertation

Maříková, J., Al Mamun, A., Al Shammari, L., Korábečný, J., Kučera, T., Hulcová, D., Kuneš, J., Malaník, M., Vašková, M., Kohelová, E., Nováková, L., Cahlíková, L., Pour, M., 2021. Structure Elucidation and Cholinesterase Inhibition Activity of Two New Minor Amaryllidaceae Alkaloids. Molecules 26, 1279.

Share of the student in the publication: isolation of new alkaloid; identification of isolated compound; writing of isolation process

Al Shammari, L., Hulcová, D., Maříková, J., Kučera, T., Šafratová, M., Nováková, L., Schmidt, M., Pulkrábková, L., Janoušek, J., Soukup, O., Kuneš, J., Opletal, L., Cahlíková, L., 2021. Amaryllidaceae Alkaloids from *Hippeastrum* x *hybridum* cv. Ferrari, and Prepararion of Vittatine Derivatives as Potential Ligands for Alzheimer's Disease. South African Journal of Botany 136, 137-146.

Share of the student in the publication: complete phytochemical study (preparation of extract, column chromatography, isolation of alkaloids), MS study of isolated alkaloids; preparation of alkaloids from biological studies, preparation of selected vittatine derivatives, writing of isolation process.

Al Shammari, L., Al Mamun, A., Koutová, D., Majorošová, M., Hulcová, D., Šafratová, M., Breiterová, K., Maříková, J., Havelek, R., Cahlíková, L., 2020. Alkaloid Profiling of *Hippeastrum* cultivars by GC-MS, Isolation of Amaryllidaceae Alkaloids and Evaluation of Their Cytotoxicity. Records of Natural Products 14, 154-159.

Share of the student in the publication: preparation of individual extracts; isolation of alkaloids, MS study of isolated alkaloids; preparation of alkaloids from biological studies, writing of isolation process.

9.2 Publications not related to dissertation

Al Shammari, L.A., Hassan, W.H.B., Al-Youssef, H.M., 2015. Phytochemical and Biological Studies of *Carduus pycnocephalus* L. Journal of Saudi Chemical. Society 19, 410-416

Hassan, W.H.B., Al-Youssef, H.M., Al Shammari, L.A., Abdallah, R.H., 2015. New Pentacyclic Triterpene Ester and Flavone Glycoside From the Biologically Active Extract of *Carduus pycnocephalus* L. Journal of Pharmacognosy and Phytotherapy 7, 45-55.

Al Shammari, L.A., Hassan, W.H.B., Al-Youssef, H.M., 2012. Chemical Composition and Antimicrobial Activity of The Essential Oil and Lipid Content of *Carduus pycnocephalus* L. Growing in Saudi Arabia. Journal of Chemical and Pharmaceutical Research 4, 1281-1287.

9.3 Conferences

9.3.1 Lectures

Al Shammari, L., Hulcová, D., Opletal, L., Cahlíková, L.: Aromatic Derivatives of Haemanthamine-Type Alkaloid Vittatine As Potential Ligands for Alzheimer's Disease. 11th Postgraduate and 9th Postdoctoral Conference, Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Czech Republic, 27-28.1.2021.

Al Shammari, L., Kuneš, J., Havelek, R., Cahlíková, L.: Isolation of Amaryllidaceae Alkaloids from *Hippeastrum* Cultivar Ferrari and Evaluation for Their Biological Activities. 10th Postgraduate and 8th Postdoctoral Conference, Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Czech Republic, 22-23.1.2020.

Al Shammari, L., Kuneš, J., Havelek, R., Cahlíková, L.: Alkaloid Profiling of *Hippeastrum* Cultivars by GC-MS, Isolation of Amaryllidaceae Alkaloids and Evaluation for Their Cytotoxic Activity. 9th Postgraduate and 7th Postdoctoral Conference, Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Czech Republic, 23-24.1.2019.

9.3.2 Posters

Al Shammari, L., Kuneš, J., Havelek, R., Cahlíková, L.: Alkaloid Profiling of *Hippeastrum* Cultivars by GC-MS, Isolation of Amaryllidaceae Alkaloids and Evaluation for Their Cytotoxic Activity. Conference of the Slovak Pharmaceutical Society (Drug Synthesis and Analysis), Faculty of Pharmacy in Bratislava, 5-6. 9. 2019

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