ALMA MATER STUDIORUM - UNIVERSITÀ DI BOLOGNA

DOTTORATO DI RICERCA IN SCIENZE FARMACOLOGICHE E TOSSICOLOGICHE, DELLO SVILUPPO E DEL MOVIMENTO UMANO

XXVI CICLO

Settore concorsuale di afferenza: 05/A1

Settore scientifico disciplinare: BIO 15

PHARMACOLOGICAL SCREENING AND BIOTECHNOLOGICAL PRODUCTION OF ALKALOIDS FROM TISSUES AND CELLS CULTURED BY PLANTS OF THE AMARYLLIDACEAE FAMILY

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Esame finale anno 2014

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INTRODUCTION

Throughout the ages humans have entrusted on Nature to cater for their basic needs, not the least of which are medicines for the treatment of a wide spectrum of diseases. Plants, in particular, have formed the basis of refined traditional medicine systems, with the earliest records, dating from around 2600 BCE, documenting the uses of approximately 1000 plant-derived substances in Mesopotamia [1]. The World Health Organization (WHO) had already estimated in 1985 that approximately 65% of the population of the world predominately relied on plant-derived traditional medicines for their basic health care, while plant products also play an important, though more indirect role in the health care systems of the remaining population who mainly reside in developed countries. A research of plant-derived pure compounds used as drugs in countries hosting WHO-Traditional Medicine Centers indicated that, of 122 compounds recognized, 80% were used for the same or correlated ethnomedical purposes and were derived from only 94 plant species [2].

Some relevant examples show how molecules that have been discovered from natural plant source can treat various aspects relating to human health care, in particular : galegine, from *Galega officinalis* L., which was the model for the synthesis of metformin and other bisguanidine-type antidiabetic drugs; ephedrine, from *Ephedra sinica*, a plant long used in traditional Chinese medicine, and the basis for the synthesis of the anti-asthma agents (beta agonists) like salbutamol and salmetrol; quinine, from the bark of *Cinchona* species (e. g., *C. officinalis*), that formed the basis for the synthesis of the commonly used antimalarial drugs, chloroquine and mefloquine; vinblastine and vincristine, isolated from the Madagascar periwinkle, *Catharanthus roseus*, important plant-derived anticancer drugs in clinical use; galantamine, a natural product discovered through an ethnobotanical lead and first isolated from *Galanthus woronowii* Losinsk., to date used to treat symptoms due to Alzheimer's disease.

The importance of natural products lies not only in their direct utilization, but also, in order to constitute the pharmacophore or synthons for new molecules, reason is that they often possess highly selective and specific biological activities based on mechanisms of action. From this perspective, the products arising from natural compounds or their direct modifications represent 50% of the new chemical entities (NCEs), as shown in Figure **1** [3].

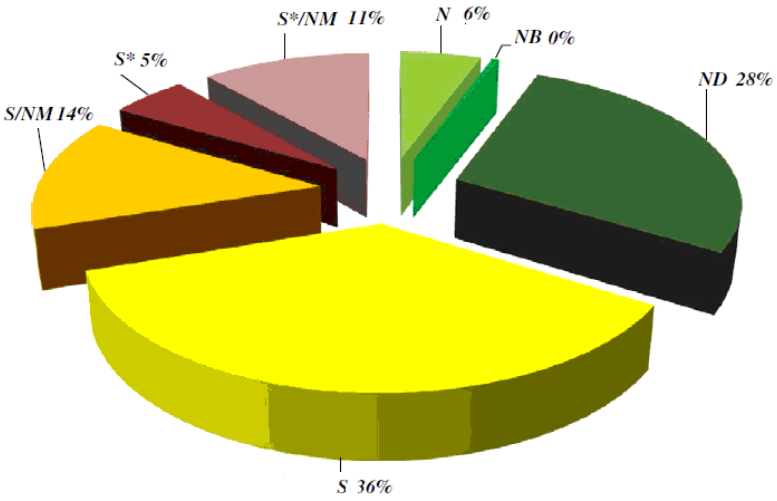


Fig. 1 Cragg et al. (2013), sources of current drugs represented by pie chart

N an unmodified natural product

NB a natural product botanical

ND amodified natural product

S a synthetic compound with no natural product conception

S/NM a synthetic compound showing competitive inhibition of the natural product substrate

*S** a synthetic compound with a natural product pharmacophore

S/NM* a synthetic compound with a natural product pharmacophore that showing competitive inhibition of the natural product substrate

The activity of natural products is mainly related to secondary metabolites from plants, compounds that can be generally classified into [4]:

- Phenols
- Compounds Sulphur-Containing
- Terpenes
- Alkaloids

Each group has different pharmacological activities, and can be divided into different classes according to own chemical structure.

Phenols, through phenolic ring structure, can play directly activity in free radical scavenger, reducing agents, prooxidants metal chelating and quencher in formation of oxygen radicals. Preclinical studies have revealed that these compounds show important

protective activities against many pathological conditions, particularly where is involved a component of oxidative stress [5].

Compounds Sulphur-Containing, derived from two main sources in the diet: those derived from the glucosinolate–myrosinase (substrate–enzyme) system found in cruciferous crops, such as cabbages, broccoli (*Brassica oleracea*) and watercress (*Nasturtium officinale*), and those derived from the alliin-alliinase system found within *Allium* crops, such as garlic (*A. sativum*), onions (*A. cepa*) and leeks (*A. porrum*). At these molecules are due the flavor and aroma, as well as many of the alleged therapeutic effects associated with these plants, including the potential chemopreventive, anti-thrombotic, hypoglycemic and hypolipidemic [6].

Terpenes represent one of the largest families of natural products, including more than 40.000 compounds. Their most important structural property is the long series of conjugated double bonds in the central part of the molecule. This kind of structure makes the molecule vulnerable to oxidation and cis-trans isomerisation [7].

Alkaloids are heterogeneous group of low molecular weight, nitrogen-containing compounds mostly derived from amino-acids and found in about 20% of plant species. As secondary metabolites, alkaloids are considered to play a defensive role in the plant against herbivores and pathogens. Due to their potent biological activity, many of the approximately 12.000 known alkaloids have been exploited as pharmaceuticals, stimulants, narcotics and poisons [8].

Since has been said previously, plants play a key role in human health care. In the same way at the development of the research of novel natural compounds, has increased the demand for more and most easily available plant sources. At the UN (United Nations) Conference held in Rio de Janeiro in 1992, have been established parameters for the preservation of biological diversity by promoting sustainable use of sources, this involves the use of natural resources in ways and times that do not lead the depletion of biodiversity. For this reason in the last decade have been increasingly investigated methods for the development of *in vitro* plant cell cultures. Advances in biotechnology for culturing plant cell cultures, today allow to obtain secondary metabolites in large quantity, bypassing problem to impoverish endangered natural sources, and by using suitable elicitors, get a possibility to increase or select the secondary metabolites production.

According to what said above, in this work particular attention was given to the study of secondary metabolites produced by some plants belonging to the Amaryllidaceae family, in the specific case isoquinoline alkaloids.

At the first instance were characterized both qualitatively and quantitatively three different plants belonging to Amaryllidaceae family, such as: *Crinum angustum* Steud., *Pancratium illyricum* L., and *Leucojum nicaeense* Ard. The alkaloids extracts obtained were separately tested against enzymes involved in specific diseases or liable in multifactorial pathologies. Considering the protection role against external bodies carried out by these metabolites in plant, extracts were also assayed against ATCC microorganisms and clinical isolates. Plants with promising pharmacological activities have been the basis for development of *in vitro* plant models.

CHAPTER 1

PLANTS OF AMARYLLIDACEAE FAMILY

1.1 INTRODUCTION

The Amaryllidaceae are a family of monocotyledonous plants, represented by 59 genera and over 850 species all over the world [9], assigned by APG (Angiosperm Phylogeny Group III) classification to the order of Asparagales. These plants have mostly tropical and subtropical distribution and their presence in temperate areas is less significant. South America (28 genera) and South Africa (19 genera) are the regions with major diversity. The Mediterranean region, being the source of numerous horticultural introductions, has only eight genera, whereas Australia has only three genera. Amaryllidaceae plants occupy many different habitats: seasonally dry places, ephemeral pools, rainforests understory, and rivers. Currently, molecular evidence places the most ancient lineages and the origin of the family in Africa [10]. The family is essentially composed by bulbous plants that in temperate areas, in the winter season losing their entire epigeal portion stems and leaves, so as to disappear completely at the sight. Though several plants belonging to this family have a considerable size, indeed in some cases can exceed 2 meters high, in other instances are less than 10 centimeters tall. Having regard to the use made of these plants in the research of novel natural compounds, the urban areas development at the expense of natural one, and in some cases their limited size, to date most of the Amaryllidaceae plants family which grow in the Mediterranean area are endangered.

1.2 THE AMARYLLIDACEAE ALKALOIDS AND THEIR BIOLOGICAL ACTIVITIES

A peculiar property of the Amaryllidaceae plants is a consistent presence of an exclusive group of alkaloids, which have been isolated from plants of all the genera of this family. In fact, the Amaryllidaceae alkaloids represent a large, and still expanding, group of isoquinoline alkaloids, the majority of which are not known to occur in any other family plants. Since the isolation of lycorine from *Narcissus pseudonarcissus* in 1877, over 300 alkaloids have been isolated from plants of this family [11], and though their structures vary significantly, these alkaloids are considered to biogenetically related. The large number of structurally different Amaryllidaceae alkaloids are classified mainly into nine skeleton types as reported by the Ghosal's model, for which the representative alkaloids are: norbelladine, lycorine, homolycorine, crinine, hemanthamine, narciclasine, tazetine, montanine and galanthamine (Fig **1.1**).

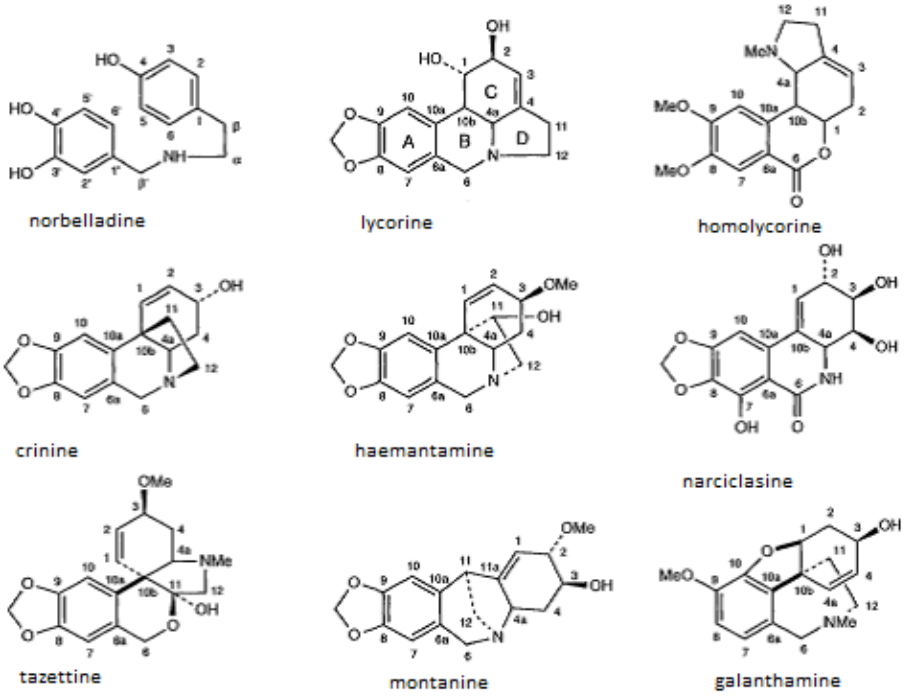


Fig. 1.1 Amaryllidaceae Alkaloid types

Plants of the Amaryllidaceae family have been used for thousands of years as herbal remedies, and the alkaloids from their extracts have been the object of active chemical

investigation for nearly 200 years. Over the past two decades many have been isolated, and screened for different biological activities by a number of research groups. A relevant issue about these alkaloids is their availability, in fact are obtained only in small quantities from natural source. For this reason, is highly developed the practice of synthesize or semi synthesize these alkaloids and their derivatives [12].

In spite of the great variety of pharmacological and biological properties exhibited by Amaryllidaceae alkaloids, only some of the activities of a reduced number of these molecules have been reported. The relationship of chemical structure and biological activity is largely unknown, and further studies are needed to explore the full therapeutic potential of these alkaloids.

Generally, activity displayed by the Amaryllidaceae alkaloids can be traced to principal groups to which they belonging:

- *Lycorine types*, with lycorine, their most representative and characteristic Amaryllidaceae alkaloids, have been reported to be strong inhibitors on parasite development and antifungal activity [13]. Additionally, the alkaloids of lycorine types are potent inducers of apoptosis in human leukemia cells, are selective inhibitors of human ovarian cancer cell, and may exert antiviral effects on several RNA- and DNA-containing viruses [14].
- *Homolycorine types*, some alkaloids of this series, such as homolycorine, being moderately active in inhibiting the *in vivo* and *in vitro* growth of a variety of tumor cells, such as Molt 4 lymphoma, HepG2 human hepatoma, and LNCaP human prostate cancer [15]. Other alkaloids homolycorine types show DNA binding activity comparable to that vinblastine [16].
- *Hemanthamine types and Crinine types*, display pronounced cell growth inhibitory activities against a variety of tumor cells, such as Rauscher viral leukemia, Molt 4 lymphoma, BL6 mouse melanoma, HepG2 human hepatoma, HeLa, LNCaP human prostate cancer [17]. Vittatine, as reported in Evidente *et al*. (2004) an alkaloids of this group, has antibacterial activity against the Gram-positive *Staphylococcus aureus* and Gram-negative *E. coli*.
- *Tazettine types*, displays weak hypotensive and antimalarial activities and interacts with DNA. Pretazettine, alkaloids of this group and labile precursor of

tazettine, shows cytotoxicity against fibroblastic LMTK cell lines and inhibits HeLa cell growth, being therapeutically effective against advanced Rauscher leukemia, Ehrlich ascites carcinoma, spontaneous AKR lymphocytic leukemia, and Lewis lung carcinoma. It is one of the most active of the Amaryllidaceae alkaloids against Molt 4 lymphoid cells [18] [19].

- *Narciclasine types*, are an antimitotic and antitumoral alkaloids, affects cell division at the metaphase stage and inhibits protein synthesis in eukaryotic ribosomes by directly interacting with the 60S subunit [20]. The peculiar effects of narciclasine seem to arise from the functional groups and conformational freedom of its C-ring, with the 7-hydroxyl group believed to be important in its biological activity [21]. Narciclasine, is one of the most important antineoplastic Amaryllidaceae alkaloids. It inhibits HeLa cell growth, and is active against a variety of tumor cells. No effect has been observed toward solid tumors. Some alkaloids of this type, such as trisphaeridine, possess high antiretroviral activities [22].
- *Montanine types*, are a group with little information. Evidente el al. (2004), reported some data about pancracine, which shows antibacterial activity against *S. aureus* and *Pseudomonas aeruginosa*.
- *Galanthamine types*, have like founder galanthamine. This alkaloid, originally isolated from *Galanthus nivalis* L. in the 1940, is a long-acting, selective, reversible and competitive inhibitor of acetylcholinesterase, in fact this product is marketed as a hydrobromide salt under the name Razadyne®, formely Reminyl®, for the treatment of Alzheimer's disease. Galanthamine has other noteworthy pharmacological actions, including an ability to amplify the nerve-muscle transfer. It is also known to cause bradycardia or atrioventricular conduction disturbance, has long been used as a reversal agent in anesthetic practice, inhibits traumatic shock, and has been patented for use in the treatment of nicotine dependence [23].

1.3 *CRINUM ANGUSTUM* STEUD.

The genus *Crinum*, belonging to Amaryllidaceae family, includes approximately 160 species present in warm temperate regions of the world [24]. Cross-hybridization of *Crinum* plants is rather widespread, mainly due to their particular interest as ornamental plants. Indeed, several species of this genus are cultivated for both ornamental and therapeutic purposes [15]. The ethnobotanical use of *Crinum* plants have been developed in different parts of the world. For instance, the bulbs of *C. asiaticum* L. were used in India as tonic, laxatives and expectorants. The *C. latifolium* L. from India was applied to treat rheumatism, abscesses, earaches and as a tonic. The roots of some *Crinum* species were used in Africa to treat urinary infections, coughs and colds, renal and hepatic conditions, sores, sexually transmitted disease and backache [25].

In this work was examined *Crinum angustum* Steud., a hybrid between *C. asiaticum* var. *asiaticum* and *C. zeylanicum* [26]. Plant at the pre-flowering stage were collected in the greenhouse of the Botanical Garden of Bologna University, and was identified by Prof. Lucia Conte. A voucher specimen (No. BOLO0507744) was deposited in the Herbarium of the University Museum System (SMA), Bologna University, Italy. In figure **1.2** is reported a picture of *Crinum angustum* Steud.

Fig. 1.2 Flower and leaves of *Crinum angustum* Steud.

1.4 *PANCRATIUM ILLYRICUM* L.

Amongst the Amaryllidaceae, the genus *Pancratium* comprises over 20 species widely spread throughout the Old World (Asia, Africa and Europe). Some of them have been studied for their potential therapeutical use [27]. Amongst these, *Pancratium maritimum* is one of the most exhaustively investigated species, and over half of all the alkaloids isolated from *Pancratium* plants have been identified in this species. Conversely, very few reports are available on *Pancratium illyricum* L., a species endemic to Sardinia, Corsica and the Tuscan archipelago [28]. In a recent work aimed at identifying new cytotoxic compounds from different Sardinian plants, a considerable activity against both human and bacterial topoisomerase has been demonstrated for three compounds isolated from bulbs of this species [29].

In the present work both bulbs and leaves of *Pancratium illyricum* L. was analysed. Plant was collected during the flowering period in the South of Sardinia (Punta San Michele, CA, Italy), and identified by Professor Mauro Ballero (University of Cagliari, Italy). A voucher specimen (CAG 1365) has been deposited in the Institute of Botany, University of Cagliari. In figure **1.3** is shown a picture of *Pancratium illyricum* L.

Fig. 1.3 Plant and flowers of *Pancratium illyricum* L. from Sardinia

1.5 *LEUCOJUM NICAEENSE* ARD.

Leucojum L. is an interesting genus from a taxonomic and cytological point of view. It consists of 12 taxa, many of which are cultivated for their ornamental value. Its distribution, almost all over Europe, includes a wide variety of habitats. The main concentration of species however, is found in the Mediterranean region, which can be considered as the center of diversity for the genus [30]. The genus *Leucojum* with *L. aestivum* L., was one of the first sources of galanthamine, and the first plant of the Amaryllidaceae family that has been studied for *in vitro* development [31].

Leucojum nicaeense Ard., plant belonging to this genus, endemic from the Maritime Alps of southern France and NW-Italy, is included in the World Red List as an endangered species and it is classified as vulnerable. For the EU it is part of the Annex II of Habitat Directive (issued by WCMC) for threatened plants [32]. For this reason, even if potential source of alkaloids to pharmacological interest, is not yet investigated in phytochemical field.

In this work have been examined, in collaboration with the Research Council for Agricultural Experimentation (CRA-FSO) of Sanremo, micropropagated plants (both *in vitro* and in greenhouse acclimatized) of *L. nicaeense* Ard.

A voucher specimen (B100341634) of original plant has been deposited in the Botanical Museum of Berlin (Germany). In figure **1.4** is shown a flower of *Leucojum nicaeense* Ard.

Fig. 1.4 Flower of *Leucojum nicaeense* Ard.

CHAPTER 2

OXIDATIVE STRESS AND ANTIOXIDANTS AGENTS

2.1 INTRODUCTION

Oxidative stress is a pathological condition caused by the breakdown of the physiological balance in organism, between production and elimination of oxidants chemical species by the antioxidant defence systems. All organisms keep a reducing environment within their cells. The cellular redox environment is preserved by enzymes which maintain the reduced state through a constant input of metabolic energy. Eventual disturbance in this normal redox state could cause toxic effects, producing peroxides and free radicals that damage all components of the cell, including proteins, lipids and DNA. The oxidant species and free radicals play important physiological roles, such as the defence against bacteria, the transmission of biochemical signals between cells, the blood pressure control. Is only their excess, generally refers to one or more classes of oxidants, to be implicated in oxidative stress.

Free radicals of importance in living organisms include hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), and peroxyl ($RO_2^{\bullet}$). Peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), singlet oxygen, and ozone (O_3) are not free radicals but can easily lead to free-radical reactions in living organisms. The term "reactive oxygen species" (ROS) is often used to include not only the radicals $OH^{\bullet}$, $RO_2^{\bullet}$, NO and $O_2^{\bullet-}$ but also the non radicals HOCl, $^1O_2$, $ONOO^-$, O_3, and H_2O_2 [33].

2.2 RADICAL FORMATION

The radicals are formed as result of acceptance or loss of an electron, and as result of the homolytic cleavage of a covalent bond: the two electrons are separated symmetrically forming two reactive intermediates each of which has an unpaired electron. The presence of electron in an aerobic environment leads to the formation, initially of the superoxide radical, then of hydrogen peroxide and finally the hydroxyl radical. The first step in the ROS generation, is represented by the oxygen acquisition of sufficient energy to reach the singlet state and the subsequent acquisition of an electron, thus giving rise superoxide anion ($O_2^{\bullet-}$).

The enzyme superoxide dismutase (SOD), adding two protons and an electron converts the superoxide anion to peroxide hydrogen (H_2O_2). $O_2^{\bullet-}$ and H_2O_2 react with each other

resulting in the formation of an oxygen molecule, a hydroxyl anion, and a radical hydroxyl ($OH^{\bullet}$) that between oxygen radicals, is the most toxic molecule and more dangerous [34]. In addition to oxygen radicals are reactive species centered on the nitrogen such as nitric oxide ($NO^{\bullet}$), nitric dioxide ($NO_2\bullet$), which as ROS, play roles in physiological conditions of extreme importance, but that if produced in excess, can cause cellular stress.

Free radicals and other reactive species not radical type, are produced in biological systems, both through processes endogenous, that following exposure to exogenous sources. For example among the physiological processes include:

- Aerobic respiration in mitochondria
- ROS formation in the cell membrane
- Peroxisome activity
- ROS production in endoplasmic reticulum

ROS production can occur also by external agents, as chemical, physical, and natural.

2.3 ANTIOXIDANT SYSTEMS

Organisms have a complex system of defence against oxidative stress, constituted by antioxidant agents both endogenous and exogenous, enzymatic or not, with different mechanism of action and chemical characteristics. Antioxidants exert their action by counteracting or by preventing the oxidation of other molecules, preventing the radical formation, or giving to these an electron. The Human organism possess defense systems extremely effective, as the catalytic activity able to sequester ROS directly or indirectly, substances that act as chemical traps against free radicals, and which restore antioxidant defences. Among these are antioxidants enzymes that catalyze the transformation or destruction of ROS, chelating compounds, and molecules that can act as a scavenger, like many vitamins, GSH (reduced glutathione), co-enzymes and polyphenols.

The main antioxidant enzymes, present in the cytosolic, are:

- Catalase (CAT), located mainly in peroxisomes, and cytosolic fraction, is an enzyme able to protect cells from the toxic effects of hydrogen peroxide (H_2O_2) by catalysing its decomposition into molecular oxygen and water, without the production of free radicals**.**

- Glutathione peroxidase (GSH-Px), a metal- enzyme containing selenium, which performs the antioxidant function at the intracellular level. Oxidative stress produces peroxides that can be reduced by using the GSH through glutathione peroxidase into water and alcohol.
- Superoxide dismutase (SOD) is a metal-enzyme that catalyzes the dismutation of the superoxide ion to molecular oxygen and peroxide hydrogen.
- The NADP(H): quinone oxidoreductase 1 (NQO1), sometimes considered an enzyme of phase II, is an enzyme belonging to the class of oxidoreductases, that catalyzes the formation of semiquinone.

Quinones are a group of very common substrates, which may have deleterious effects, such as the ability to attack nucleophiles and generate reactive oxygen species, which are reduced with a mechanism of transfer of ions hydride to generate the corresponding derivative hydroquinonic. The NQO1 has also an important role in the metabolism of endogenous quinones such as vitamin E and ubiquinone. For example the reduction of ubiquinone to work of NQO1 regenerates ubiquinol, which possesses strong antioxidant properties [35].

2.4 OXIDATIVE STRESS AND RELATED DISEASES

Oxidative stress refers to all changes which produced in tissues, cells and biological macromolecules, when following an alteration of the balance between intracellular antioxidant defenses and pro-oxidant elements, with accumulation of radical species. The wide variety of mechanisms that can be activated as a consequence of redox imbalance can significantly contribute to the development of numerous pathological conditions, in particular, cancers, inflammatory pathologies, diabetes, ischemia, cardiovascular disease and neurodegenerative diseases. However, it is still unclear whether oxidative stress is among the main causes, or between the events that occur during the pathological process and that contribute to the progression of the disease. For example, in Parkinson's disease, is unlikely that oxidative stress is the primary event, of the degenerative process that leads to the depletion of dopaminergic neurons, but it is certain, however, that during the disease to occur oxidative phenomena involved substantially into progression of neuronal damage. ROS appear to be involved in many inflammatory processes, which, although not produce, certainly characterize various diseases associated with aging.

CHAPTER 3

ALZHEIMER DISEASE

3.1 INTRODUCTION

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder associated with a rapid cognitive decline with an average of survival of 5-10 years after diagnosis. Furthermore, AD clearly differs from the normal aging in that it causes dramatic loss of synapsis, neurons and brain activity in specific anatomical regions, and results in massive atrophy and gliosis.

The factors that cause some individuals to depart from the relatively benign of normal aging brain and instead undergo the pathological cascade that leads to AD are unknown [36].

Although memory loss is usually the initial and most prominent problem, deficits in cognitive domains other than memory can occur in the early stages of disease in some patients. Final deterioration leads to a bedridden, mute, incontinent, and unresponsive state, which mimics the persistent vegetative state.

Pathologically AD is characterized by the presence of two insoluble protein aggregates, senile plaques formed from the peptide β-amyloid (Aβ) and neurofibrillary tangles composed of hyperphosphorylated tau protein [37]. In rare familial AD, the cause of disease is autosomal dominant mutations in Aβ precursor protein (*APP*) or the Aβ-producing enzymes presenilins (*PSEN1* or *PSEN2*), which are all thought to lead to increased levels of aggregated Aβ [38]. Likewise, mutations in tau (*MAPT*) that predispose it to aggregation can cause specific diseases that involve profound neurodegeneration and dementia. Thus, like in other neurodegenerative diseases such as Huntington's disease (HD) and Parkinson's disease, the formation of toxic insoluble aggregates seems to be a key pathogenic step. However, it is not known why these Aβ and tau aggregates accumulate in AD patients nor how they contribute to neuronal dysfunction, particularly for Aβ deposits, which can often be found in the brains of elderly non-demented subjects [39].

An important goal of AD research is to identify interventions that maintain brain function, potentially by inhibiting the formation or improving the clearance of neurotoxic aggregates, or by promoting resistance to, or recovery from, damage.

The clinical presentation of AD is heterogeneous and insidious, and the psychological and financial effects of AD on caregivers and family members are significant.

3.2 THERAPY IN ALZHEIMER'S DISEASE

More than a decade after the first approval of the use of Acetylcholinesterase inhibitor on patients with Alzheimer's disease, still not have a single treatment or combination therapy that can effectively stop or reverse the progression of such neurodegenerative disease.

Probably there is not one single cause, but several factors are important to describe the etiology of the disease. Therefore, combination of compounds, which act at more than one target site, could be useful for AD treatment.

To date, there are only 5 medications approved by Food Drug Administration (FDA) to treat AD.

They include 4 Acethylcholinesterase inhibitor (AChEIs), and one N-methyl-D-aspartate (NMDA) antagonist, molecule that regulates the activity of glutamic acid receptor.

3.3 CHOLINESTERASE INHIBITORS

The cholinergic hypothesis Of AD concludes that cholinergic system in the basal forebrain are affected early in the disease process including loss of the acetylcholine neurons, loss of enzymatic function for acetylcholine synthesis and degradation, resulting in memory loss as well as deterioration of other cognitive and noncognitive functions such as neuropsychiatric symptoms. A strategy to enhance the cholinergic transmission by using AChEIs to delay the degradation of acetylcholine between the synaptic cleft was then proposed. In 1993 the first FDA approved AChEIs, tacrine boomed out but it was no longer use because of its high prevalence of hepatotoxicity. FDA approved another three AChEIs: donepezil (1996), rivastigmine (2000), and galanthamine (2001) in the following years.

These drugs have been regarded as the standard and first-line treatment for of AD. Systemic reviews, showed benefit on cognitive functions, activities of daily living (ADL), and global functions for patients mild to moderate AD.

Certainly these drugs do not lead to the cure of AD, but to continue to identify AChEIs with fewer adverse effects and capable of alleviate symptoms of AD also in advanced stage, could represent a valid area to study, and Amaryllidaceae plants are good sources of molecules with this activity.

CHAPTER 4
MATRIX METALLOPROTEINASES AND REGULATION OF CELL BEHAVIOR

4.1 INTRODUCTION

Extracellular proteinases are required for numerous developmental and disease-related processes. The ability to degrade extracellular proteins is essential for any individual cell to interact properly with its immediate surroundings and for multicellular organisms to develop and function normally. On this basis, a family of related enzymes has been identified in species from hydra to humans and collectively called matrix metalloproteinases (MMPs), because of their dependence on metal ions for catalytic activity, their potent ability to degrade structural proteins of the extracellular matrix (ECM), and specific evolutionary sequence considerations that distinguish them from other closely related metalloproteinases.

Essentially Matrix metalloproteinases (MMPs) constitute a multigene family of over 25 secreted and cell surface enzymes that process or degrade numerous pericellular substrates. Their targets include other proteinases, proteinase inhibitors, clotting factors, chemotactic molecules, latent growth factors, growth factor–binding proteins, cell surface receptors, cell-cell adhesion molecules, and virtually all structural extracellular matrix proteins. Thus MMPs are able to regulate many biologic processes and are closely regulated themselves [40].

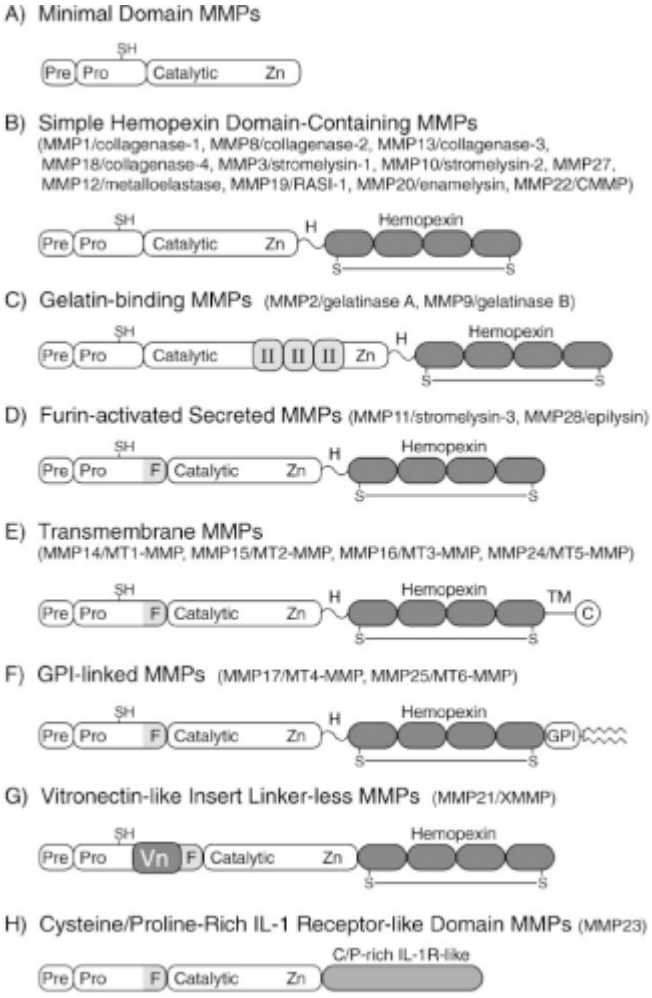
In addition to their ECM substrates, MMPs also cleave cell surface molecules and other pericellular non-matrix proteins, thereby regulating cell behavior in several ways [41]. Thus like the many proteins they modify, the MMPs influence diverse physiologic and pathologic processes, including aspects of embryonic development, tissue morphogenesis, wound repair, inflammatory diseases, and cancer [42].

At present, 25 vertebrate MMPs and 22 human homologues have been identified and have been classified according to substrate specificity and are often referred to as: collagenases, gelatinases, stromelysins, martilysins, and membrane-type MMPs. All contain Zn^{2+} at the catalytic site as well as an additional zinc ion and calcium ions for stability. Individual MMPs are referred to by their common names or according to a

sequential numeric nomenclature reserved for the vertebrate MMPs. In addition, they are often grouped according to their modular domain structure. In this regard, all MMPs have an N-terminal signal sequence (or “pre” domain) that is removed after it directs

synthesis to the endoplasmic reticulum. Thus most MMPs are secreted; however, six display transmembrane domains and are expressed as cell surface enzymes. The pre domain is followed by a propeptide “pro” domain that maintains enzyme latency until it is removed or disrupted, and a catalytic domain that contains the conserved zinc-binding region. The catalytic domain dictates cleavage-site specificity through its active site cleft, through specificity sub-site pockets that bind amino acid residues immediately adjacent to the scissile peptide bond, and through secondary substrate-binding exosites located outside the active site itself [43]. In figure **4.1** are shown domain structure in different kinds of MMPs.

Fig. 4.1 Domain structure in different kinds of MMPs



Most of the MMPs are synthesized as inactive latent enzymes. Conversion to the active enzyme is generally mediated by activator systems that include plasminogen activator or the pro-hormone convertase, furin. MMP activity is regulated by a group of endogenous proteins, called, tissue inhibitor of metalloproteinases (TIMPs) that bind to active and alternative sites of the activated MMP.

The TIMPs represent a family of at least four 20–29-kDa secreted proteins (TIMPs 1–4) that reversibly inhibit the MMPs in a 1:1 stoichiometric fashion. Individual TIMPs differ in their ability to inhibit various MMPs. In addition, the TIMPs differ in terms of their gene regulation and tissue-specific patterns of gene expression [44].

To date, no TIMP receptors have yet been identified, suggesting that TIMPs may act as decoys for various signaling molecules. TIMPs are not the only endogenous MMP inhibitors. Indeed, α2-macroglobulin is a major endogenous inhibitor of the MMPs, and

its importance may have been overlooked due to the recent emphasis placed on the TIMPs. Because α2-macroglobulin is an abundant plasma protein, it represents the major inhibitor of MMPs in tissue fluids, whereas TIMPs may act locally. Moreover, α2-macroglobulin plays an important role in the irreversible clearance of MMPs, whereas TIMPs inhibit MMPs in a reversible manner.

4.2 MMPs AND DISEASE

Studies using relevant disease models in MMP-deficient animals have demonstrated the contribution of MMPs to disease processes. In the cardiovascular area, MMPs have been strongly associated with aneurysms [45], with atherosclerotic plaque rupture [46], with myocardial infarction, left ventricular remodeling and ultimate cardiac rupture [47], as well as cerebral ischemia events [48]. MMP expression is raised in multiple tumor types and mostly, these increases correlate with decreased survival. In both rheumatoid- and osteo-arthritis, MMPs are considered to be significantly responsible for the matrix degradation that characterizes these diseases [49]. Respiratory disorders including idiopathic pulmonary fibrosis, asthma, emphysema, and acute respiratory distress syndrome [50],are also strongly associated with MMP activity.

Is important to emphasize that in some pathologies the correct MMP activity leads to a positive resolution of the disease, while in others, this result is achieved by inhibiting the activity of MMPs. Rather than categorizing specific MMPs as “good” or “bad”, it is more helpful to consider activities in particular contexts. For instance expression studies in humans have shown protective roles of certain MMPs. In particular, high levels of MMP12 correlate with better prognosis in several tumor types including hepatocellular and colorectal carcinoma. This is in direct contrast to the destructive role of MMP12 in emphysema where the elastase-degrading activity of MMP12 clearly contributes to pathophysiology. By generalizing it can be stated that in chronic diseases, inflammatory, and in some tumors, leads to disease an hyperactivity of MMPs.

Is precisely on this type of disorders that will be tested extracts obtained in this work, using the collagenase enzyme from *Clostridium histolyticum*, as starting model for MMPs inhibition screening; in fact, scientific literature sees in the natural extracts, a great source of compounds with inhibitory activity on matrix metalloproteinase [51] [52].

CHAPTER 5
TYROSINASE ENZYME

5.1 INTRODUCTION

Tyrosinases also known as polyphenol oxidase (PPO), is a copper-containing enzyme widely distributed in microorganism, animals, and plants, which catalyze the oxidations of both monophenols (cresolase or monophenolase activity) and o-diphenols (catecholase or diphenolase activity) into reactive o-quinones. The term tyrosinase refers to its typical substrate, tyrosine. Both tyrosinase activities appear to have broad substrate specificities, although the enzyme has a higher affinity for the L-isomers of the substrates than for the corresponding D-isomers. The first biochemical investigations were carried out in 1895 on the mushroom *Russula nigricans*, whose cut flesh turns red and then black on exposure to air. Since this study, the enzyme has been found widely distributed throughout the phylogenetic scale from bacteria to mammals. The best-characterized tyrosinases are derived from *Streptomyces glausescens*, the fungi *Neurospora crassa* and *Agaricus bisporus*. In fungi and vertebrates, tyrosinase catalyzes the initial step in the formation of the pigment melanin from tyrosine.

The notable feature observed in tyrosinases from different sources is that the central copper-binding domain is conserved, which contains strictly conserved amino acid residues, including three histidines. One tyrosinase molecule can contain two copper atoms, and each atom of the binuclear copper cluster is ligated to three histidines. In the formation of melanin pigments, three types of tyrosinase (oxy-, met-, and deoxytyrosinase) with different binuclear copper structures of the active site are involved [53].

Melanogenesis is initiated with the first step of tyrosine oxidation to dopaquinone catalyzed by tyrosinase. This first step is the rate-limiting step in melanin synthesis because the remainder of the reaction sequence can proceed spontaneously at a physiological pH value. The subsequent dopaquinone is converted to dopa and dopachrome through auto-oxidation.

Dopa is also the substrate of tyrosinase and oxidized to dopaquinone again by the enzyme. Finally, eumelanin are formed through a series of oxidation reactions from

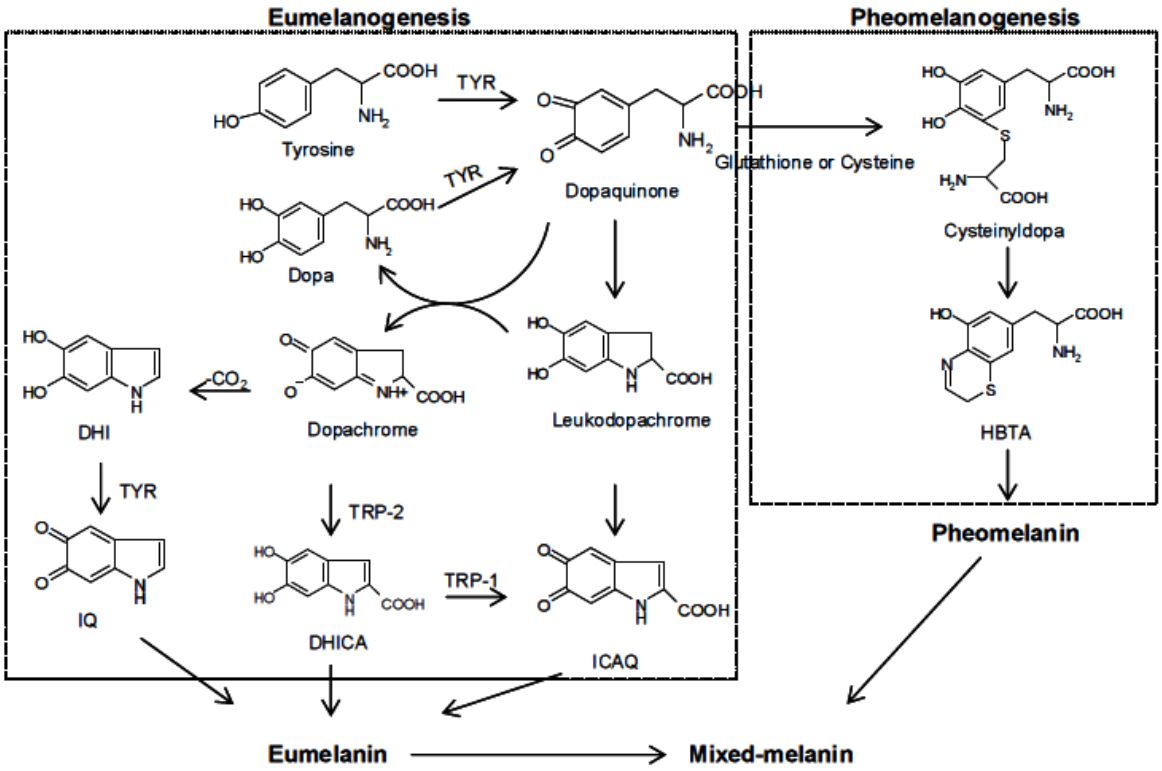
dihydroxyindole (DHI) and dihydroxyindole-2-carboxylic acid (DHICA), which are the reaction products from dopachrome. In the presence of cysteine or glutathione, dopaquinone is converted to cysteinyldopa or glutathionyldopa. Subsequently, pheomelanin is formed. In addition to eumelanin and pheomelanin, other "melanin" relying on phenolic monomers different from tyrosine is termed allomelanin. The browning phenomenon in fruit and fungi is also usually related to oxidative polymerization, conceptually similar to melanogenesis.

The main difference resides in the fact that allomelanin substantially does not contain dopaquinone-derived motifs as the main monomers in its structure and, on the contrary, is based on other quinoid building blocks [54].

Melanin plays an important role in protecting human skin from the harmful effects of sun UV radiation. Melanin also determines our phenotypic appearance.

In Figure **5.1** is shown biosynthetic pathway of melanin.

Fig. 5.1 Biosynthetic pathway of melanin. TYR, tyrosinase; TRP; tyrosinase related protein; dopa, 3,4-dihydroxyphenylalanine; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DHI, 5,6-dihydroxyindole; ICAQ, indole-2-carboxylic acid-5,6-quinone; IQ, indole5,6-quinone; HBTA, 5-hydroxy-1,4-benzothiazinylalanine.



5.2 ROLE OF TYROSINASE IN MAN AND NATURE

Melanogenesis has been defined as the entire process leading to the formation of dark macromolecular pigments, i.e., melanin. Melanin is formed by a combination of enzymatically catalyzed and chemical reactions. It is a heterogeneous polyphenol-like biopolymer with a complex structure and color varying from yellow to black. The color of mammalian skin and hair is determined by a number of factors, the most important of which is in fact, the degree and distribution of melanin pigmentation. Melanin is secreted by melanocyte cells distributed in the basal layer of the dermis. The role of melanin is to protect the skin from ultraviolet (UV) damage by absorbing UV sunlight and removing reactive oxygen species (ROS). Various dermatological disorders result in the accumulation of an excessive level of epidermal pigmentation. These hyperpigmented lentigenes include melasma, age spots and sites of actinic damage [55].

Great interest has been shown in the involvement of melanins in malignant melanoma, the most life-threatening skin tumors. Is evident, like tyrosinase play a key role both correct pigmentation of skin and in development of melanoma. This enzyme in fact, shows antiproliferative and apoptotic activity against malignant melanocytes.

In the food industry, tyrosinase is a very important enzyme in controlling the quality and economics of fruits and vegetable. Tyrosinase catalyzes the oxidation of phenolic compounds to the corrisponding quinones and is responsible for the enzymatic browning of fruits and vegetable. In addition to the undesiderable color and flavor, the quinone compounds produced in the browning reaction may irreversibly react with the amino and sulfhydryl groups of proteins. The quinone-protein reaction decreases the digestibility of the protein and the bioavailability of essential amino acids, including lysine and cysteine [56].

At last, tyrosinase, plays an important role in the developmental and defensive functions of insects; is interested in fact in the insect molting, through the formation of the cuticle leads larva to insect [57].

5.3 TYROSINASE INHIBITORS AND THEIR IMPORTANCE

A number of tyrosinase inhibitors from both natural and synthetic sources have been identified. However, the definition of "tyrosinase inhibitor" is sometimes misleading: many authors use that terminology in reference to melanogenesis inhibitors, whose action mainly resides in some interference in melanin formation, regardless of any direct inhibitor/enzyme interaction. Many putative inhibitors are examined in the presence of tyrosine or dopa as the enzyme substrate, and activity is assessed in terms of dopachrome formation. Thus, experimental observation of the inhibition of tyrosinase activity can be accomplished by one of following:

1. Reducing agents, which causing chemical reduction of dopaquinone such as ascorbic acid, avoid dopachrome and melanin formations. For this reason are used as a melanogenesis inhibitor for their capacity to reduce back o-dopaquinone to dopa.

2. *o*-Dopaquinone scavenger such as most thio-containing compounds, which are well-known melanogenesis inhibitors and react with dopaquinone to form colorless products. The melanogenetic process is therefore slowed until all the scavenger is consumed, and then it goes at its original rate.
3. Alternative enzyme substrates such as some phenolic compounds, whose quinoid reaction products absorb in a spectral range different from that of dopachrome. When these phenolics show a good affinity for the enzyme, dopachrome formation is prevented, and they could be mistakenly classified as inhibitors.
4. Nonspecific enzyme inactivators such as acids or bases, which non-specifically denature the enzyme, thus inhibiting its activity.
5. Specific tyrosinase inactivators such as mechanism-based inhibitors, which are also called suicide substrates. These inhibitors can be catalyzed by tyrosinase and form covalent bond with the enzyme, thus irreversibly inactivating the enzyme during catalytic reaction. They inhibit tyrosinase activity by inducing the enzyme catalyzing "suicide reaction."
6. Specific tyrosinase inhibitors. The compounds bind reversibly to tyrosinase and reduce its catalytic capacity.

Among the six types of compounds described above, only specific tyrosinase inactivators (5) and inhibitors (6) are regarded as "true inhibitors," which actually bind to the enzyme and inhibit its activity [54].

The discovery and characterization of new tyrosinase inhibitors are useful for their potential applications, and also because there are few "true inhibitiors" available. The fields of interest for tyrosinase inhibitors is clear that are various: improving food quality and nutritional value, controlling insect pests, preventing pigmentation disorders and other melanin-related health problems in human beings.

CHAPTER 6 :
THE PROBLEM OF MULTIDRUG RESISTANCE (MDR) AND NOSOCOMIAL INFECTIONS

6.1 INTRODUCTION

According to the World Health Organization, infectious diseases are a significant cause of morbidity and mortality worldwide, accounting for approximately 50% of all deaths in tropical countries. Infectious and parasitic diseases remain primary causes of pediatric mortality in developing countries, partly as a result of the HIV/AIDS epidemic [58]. Despite the progress made in the understanding of microbiology and the control of microorganisms, sporadic incidents of epidemics due to drug resistant microorganisms and hitherto unknown disease causing microbes pose an enormous threat to public health. Such negative health trends call for a new global initiative for the development of new strategies for the prevention and treatment of infectious disease [59].

Proposed solutions outlined by the Center for Disease Control as a multipronged approach include: prevention, improved monitoring, and the development of new treatments that exceed the resistance bacterial.

It was just a few years after the introduction of penicillin that scientists began to notice the emergence of a penicillin resistant strain of *Staphylococcus aureus*, a common bacterium that makes up part of normal human bacterial flora.

Since then, resistant strains of gonorrhoea, dysentery causing *Shigella* (a major cause of premature death in developing countries) and *Salmonella* followed in the wake of *S. aureus.* From the initial case of resistant *Staphylococcus aureus*, the problem of antimicrobial resistance has grown into a serious threat to public with economic, social, and medical implications that are global in scope and cross all environmental and ethnic boundaries [60].

National Nosocomial Infection Surveillance (NNIS) System data demonstrate a steady increase in the incidence of nosocomial infections caused by methicillin-resistant *S. aureus* (MRSA) among ICU (intesive Care Units) patients over time. MRSA now accounts for more than 60% of *S. aureus* isolates in USA hospital ICUs. In addition, the medical issues of these infected patients have become more complex as a result of sophisticated medical system, and the parallel development of infections at already present bacterial resistance can lead to death of the patients [61].

In addition to the problem of multi-drug resistance (MRD), should consider the increasing development of nosocomial infections. One of the primarily responsible of hospital infections, is the *Candida* with its spp.

Candida spp. are an important cause of nosocomial bloodstream infections (BSIs). The incidence of BSIs caused by Candida spp. has risen in the past 20 years [62], and *Candida* spp. are currently the fourth leading cause of nosocomial BSI in the USA. Mortality rates range between 29% and 76%, with an attributable mortality high as 49% [63]. Several *Candida* spp. exhibit reduced susceptibility to fluconazole and others antifungal drugs, so, also in this case is important the development of new treatments.

6.2 MEDICINAL PLANTS AND TREATMENT OF MICROBIAL INFECTIONS

Naturally derived compounds have made considerable contributions to human health and well-being, and have been a source of inspiration for novel drug development. There are thousands of published scientific papers from around the globe describing the antimicrobial activities of plants extract. The majority of the plants had activity against a range of bacteria, fungi and Mycobacterium. Many of the plant species with antimicrobial activity were native to tropical countries, due to the extraordinary biodiversity of these places.

Is important to note that, while many of the medicinal plant extracts purported to have antibacterial activity have only been subjected to *in vitro* screening, and the vast majority of these extracts have never been tested rigorously in animal models or controlled clinical trials [64].

In this study, the extracts and pure compounds tested, were analyzed both as regards the ATCC strains and on clinical isolated, with the aim to be able to approach the data obtained as much to a real result.

CHAPTER 7 :

PLANT CELL CULTURE SYSTEMS: A POTENTIAL RENEWABLE SOURCE OF VALUABLE MEDICINAL COMPOUNDS

Many higher plants are major sources of natural products used as pharmaceuticals, agrochemicals, and food additives. The search for new plant derived chemicals should thus be a priority in current and future efforts toward sustainable conservation and rational utilization of biodiversity. In the search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites. Cell suspension culture systems could be used for large scale culturing of plant cells from which secondary metabolites could be extracted. The advantage of this method is that it can ultimately provide a continuous, reliable source of natural products [65].

Discoveries of cell cultures capable of producing specific medicinal compounds at a rate similar or superior to that of intact plants have accelerated in the last few years. New physiologically active substances of medicinal interest have been found by bioassay. It has been demonstrated that the biosynthetic activity of cultured cells can be enhanced by regulating environmental factors, well as by artificial selection or the induction of variant clones. Some of the medicinal compounds localized in morphologically specialized tissues or organs of native plants have been produced in culture systems not only by inducing specific organized cultures, but also by undifferentiated cell cultures. The major advantages of a cell culture system over the conventional cultivation of whole plants are: 1) Useful compounds can be produced under controlled conditions independent of climatic changes or soil conditions; 2) Cultured cells would be free of microbes and insects; 3) The cells of any plants, tropical or alpine, could easily be multiplied to yield their specific metabolites; 4) Automated control of cell growth and rational regulation of metabolite processes would reduce of labor costs and improve productivity; 5) Organic substances are extractable from callus cultures.

Advances in tissue culture, combined with improvement in genetic engineering, specifically transformation technology, has opened new avenues for high volume production of pharmaceuticals, nutraceuticals, and other beneficial substances [66].

CHAPTER 8 :

AIM OF THE STUDY

Historically, man has used plants to supply almost all of his basic needs, and plants have always played a central role in traditional systems of medicine for the prevention and treatment of disease around the world.

In the development of new drugs, the role of natural products has been two fold: 1) they may become the base for the development of a medicine, or, 2) a phytomedicine to be used for the treatment of disease.

On these basis, in this study, are analysed plants extract of Amaryllidaceae family, plants that are known to contain secondary metabolites of high pharmacological profile, since they have been proven to possess antibacterial, antifungal, antiprotozoal, antitumoral, and acetylcholinesterase inhibitory activities [67].

Since these plants have never been studied, the purpose is:

- to characterize with phytochemical studies the alkaloid extracts.
- In the event that encounter compounds not yet classified, carry out a complete characterization, by establishing the properties of the compounds.
- To test extracts on pharmacological targets, that representing both degenerative and chronic diseases.
- Achieve a development of *in vitro* plant cell cultures, with the goal of obtaining *in vitro* production of alkaloids, equivalent to that of plant.

The *in vitro* development will be made, not only for protection of the species biodiversity because the plants analyzed in this study are endangered, but also because in the case that plant will grow *in vitro* and will produce in the same manner of the original one, will be controlled the production of secondary metabolites with addition of elicitors.

The pharmacological targets chosen for screening of the extracts activity, are associated with a wide range of disorders or diseases, not only, their activity can also be extended to fields such as cosmetics, food and agrochemical; just think in fact of the applicability that could have the inhibition of enzymes such as: Collagenase, Tyrosinase and Acetylcholinesterase.

Not least, the extracts will be tested on Multi-Drug Resistant (MDR) microorganism, considering the original protective role against attacks from external agents that have the alkaloids in plants.

With the development of MDR and the consequent hospital infections, which daily involve discomfort and increased risk on the patient's health with a consequent increase in health spending, the development of new molecules with antimicrobial activity and not subject to bacterial resistance could open new ways for the development of new drugs.

CHAPTER 9 :

MATERIALS AND METHODS

9.1 EXTRACTION AND ISOLATION OF ALKALOIDS

The extracts of three Amaryllidaceae plants of *C. angustum*, *P.illyricum* and L. nicaeense (all 6 micropropagated plants) were separately obtained from fresh plants, as follows: were crushed separately and exhaustively extracted with MeOH at room temperature for 72 h. The extracts were evaporated under reduced pressure. These crude extracts were acidified by dissolving in 100 mL H_2SO_4 1% (v/v) and neutral material was removed with *n*-hexane (6 x 100 mL) and $CHCl_3$ (4 x 100 mL). The acidified solutions were then basified with 25% NH_4OH up to pH 9-10 and extracted with $CHCl_3$ (4 x 100 mL) to give the chloroformic extract containing alkaloids. The extracts were dried with anhydrous Na_2SO_4, filtered and completely dried under reduced pressure. As regard leaf chloroformic extract of *P. illyricum* was subjected to vacuum liquid chromatography (VLC) [68] using a silica gel 60 A (6-35 μ) column with a diameter of 1 cm and height of 4 cm. Alkaloids were eluted using *n*-hexane gradually enriched with EtOAc, then gradually enriched with $CHCl_3$ and finally with a mixture of EtOAc and $CHCl_3$ gradually enriched with MeOH. Fractions of 10 mL were collected (200 in total), monitored by TLC (Dragendorff's reagent, UV 254 nm) and combined according to their profiles. Four main fractions were obtained and subjected to preparative TLC (20 cm x 20 cm x 0.25 mm, silica gel 60F254). 11α-hydroxy-*O*-methylleucotamine (7 mg) was obtained in major quantities from fractions *40-53* (eluted from VLC with *n*-Hexane-EtOAc, 64:36 to 56:44) through preparative TLC (EtOAc-$CHCl_3$-CH_3OH 4:1.5:0.5 + 25% ammonia).

9.2 GC-MS ANALYIS

The extracts of *C. angustum*, *P. illyricum* and *L. nicaeense* (all 6 micropropagated plants) obtained separately were analyzed qualitatively by GC-MS analysis starting from a mother solution of 300 μg/mL.

EIMS were obtained on a GC-MS Hewlett-Packard 6890 + MSD 5975 operating in EI mode at 70 eV. A HP-5 MS column (30 m × 0.25 mm × 0.25 μm) was used. The temperature program was: 100-180 ºC at 15 ºC min^{-1}, 1 min hold at 180 ºC, 180-300 ºC at 5 ºC min^{-1} and 1 min hold at 300 ºC. Injector temperature was 280 ºC. The flow rate

of the carrier gas (helium) was 0.8 mL min^{-1}. In most cases the split ratio was 1:20, but with more diluted samples a split ratio of 1:5.

9.3 HPLC-DAD ANALYSIS

The quantitative analysis of *C. angustum*, *P. illyricum* and *L. nicaeense* (all 6 micropropagated plants) obtained separately were carried out using an HPLC system (Jasco Corp., Tokyo, Japan) consisting of a PU-1580 pump, an LG-1580-02 ternary gradient unit, a DG-1580-53 three-line degasser, and a diode array (DAD) detector MD-2018 Plus operating at 280, 240 and 220 nm, linked to an autosampler (2055 Plus). A Spherisorb C18 column (5 μm, ODS2, 4.6 × 250 mm, Waters Corp., Milford, MA, USA) was used with a flow rate of 0.7 ml/min and elution was performed under 27 °C temperature. The mobile phase was made up of acetonitrile:ammonium acetate 80 mM pH 6.0 (30:70). Data were acquired and processed by the Chromnav Chromatography Data system software (Jasco).

9.4 GENERAL

All chemical products used were provided by Sigma Aldrich. UV spectra were obtained on a DINKO UV2310 instrument and IR spectra were recorded on a Nicolet Avatar 320 FT-IR spectrophotometer. Biological assays were performed using a microplate reader, Victor X3 Perkin-Elmer (Perkin-Elmer Inc., Boston, MA, USA) and dataanalyzed with the software Work Out 2.5 or in a Jasco V-530 spectrophotometer (Jasco Europe, Cremella, Italy). Statistical analyzes were obtained from software GraphPad Prism 5.

9.5 NMR ANALYSIS

Compound isolated from the leaves extract of *P. illyricum* was subjected to NMR analysis as follows: NMR spectra (one and two dimensional) were recorded in a Varian VXR 500 MHz, instrument using CDCl3 as the solvent and TMS as the internal standard. Chemical shifts were reported in δ units (ppm) and coupling constants (*J*) in Hz.

9.6 CYTOTOXICITY ASSAY

The human embryo lung fibroblast (HEL) were grown in DMEM (Gibco®, Life technologies) supplemented with 10% foetal calf serum (Lonza, BioWhittaker), 10U penicillin and 50 μg/ml streptomycin at 37°C. For each set of experiments, cells were seeded into 96-well plates at 10^4 cells/well and incubated with 2-fold serial dilutions starting from 2.50 mg/mL of the extract sample; there were positive controls containing cells in regular medium and solvent controls containing cells in 2-fold serial dilutions of DMSO (from 6.25 %). Cells were grown for 24 h at 37°C, then 50 μL of XTT labeling mixture (Cell Proliferation kit II, XTT, Roche) were added to cells, followed by a 6-h incubation allowing formazan production. Finally, OD was measured at 450/630 nm by using the microplate reader. The cytotoxic activity was expressed as the IC_{20} (20% inhibitory concentration) after a 24 h-incubation period, relative to the control.

9.7 ANTIOXIDANTS ASSAYS

9.7.1 DPPH AND ABTS ASSAY

The DPPH assay was performed according to the method of Venditti et al. (2013) [69], with some modifications. Stock solutions of plants extract were prepared in water/DMSO (90-10) to obtain different final concentrations (from 5 to 200 μg/mL in the assay) to calculate the IC_{50} value. One and a half milliliters of a 0.05 mM DPPH methanol solution was added to different concentrations of plants extract and allowed to react at room temperature. The assay was performed in a final volume of 2 mL. After 20 min, the absorbance (Abs) values were measured at 517 nm and converted into percentage antioxidant activity using the following formula:

Scavenging capacity% = [1 (Abs sample /Abs control) 100]

The DPPH solution plus methanol was used as a negative control, whereas Trolox (TR) at different concentrations (from 5 to 40 μM) was used as reference antioxidant compound. The IC_{50} values were calculated by logarithmic regression of plots, where the x-axis represents the individual plants extract or reference compound concentrations and the y-axis the average percentage of scavenging capacity.

The ABTS assay was performed according to the method of Venditti et al. (2013) with some modifications. ABTS•+ radical was generated by mixing a 2 mM ABTS solution with 7 mM $K_2S_2O_8$ and incubating in the dark for 24h at room temperature. Before usage, the ABTS •+ solution was diluted (1−25 mL methanol) to obtain an Abs value of 0.7 at 734 nm. Upon addition of 1 mL of the diluted ABTS•+ solution to 10 μL of reference compound or plants extract stock solutions (from 5 to 200 μg/mL), the Abs at 734 nm was recorded after 1 min. The final TEAC value of plants extract was calculated by comparing ABTS•+ decolorization with that of Trolox. The IC_{50} value was calculated as described above.

9.7.2 β-CAROTENE BLEACHING ASSAY

Prevention of the autoxidation of emulsified linoleic acid was determined by modifying method of Venditti et al. (2013). Briefly, 20 μL of different plants extract or reference compound stock solutions (50, 100, 150, and 200 μg/mL) was added to the microplate wells (Costar 3599) in duplicate. Then, 10 μL of linoleic acid, 47 μL of Tween 40, and 2.5 mL of β-carotene (2 mg/mL in chloroform) were placed in a flask. After removal of chloroform with nitrogen gas, 22,5 mL of distilled hot water (50 °C) and 2.5 mL of 0.1 M sodium phosphate buffer (pH 6.8) were added to the same flask and shaken well. An amount of 0.2 mL of the linoleic acid−β- carotene emulsion were transferred rapidly to each well, kept under constant temperature (50 °C), and the Abs at 490 nm monitored for 60 min. H2O (20 μL) and reference compound (20 μL) at different concentrations (0.25, 0.5, 1, 1.5, and 2.5 mM) were used as negative and positive controls, respectively. The difference in Abs at 50 and 5 min (Δ = Abs 50 min − Abs 5 min) was calculated. Results are expressed in terms of percentage bleaching inhibition of the initial linoleic acid−β-carotene emulsion by the test samples according to the following equation:

$$\%\text{bleaching inhibition} = [1-(\Delta\text{abs sample} / \Delta \text{ Abs negative control}) 100]$$

IC_{50} values were calculated by logarithmic regression in terms of micromolar or μg/mL. The antioxidant activity was also expressed as TEAC, by comparing IC_{50} values obtained for plants extract with that of Trolox (TR).

9.8 STUDY OF THE ENZYMATIC INHIBITION ACTIVITY

9.8.1 INHIBITION OF THE ACETYLCHOLINESTERASE (ACHE) ENZYME

The assay for measuring AChE activity was performed as described by López et al. (2002). In brief, 50 µL of AChE in buffer phosphate (8 mM K_2HPO_4, 2.3 mM NaH_2PO_4, 0.15 mM NaCl, 0.05% Tween 20, pH 7.6) and 50 µL of the sample dissolved in the same buffer were added to the wells. The plates were incubated for 30 min at room temperature before the addition of 100 µL of the substrate solution (0.1 M Na_2HPO_4, 0.5 M 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 0.6 mM acetylthiocholine iodide, pH 7.5). The absorbances were read in a microplate reader Victor X3 Perkin Elmer (Perkin Elmer Inc., Massachusetts USA) at 405 nm after five minutes. Galanthamine hydrobromide was used as a positive control. The IC_{50} of bulbs and leaves extract of *P. illyricum*, 11α-hydroxy-*O*-methylleucotamine and galanthamine hydrobromide was determined in triplicate and the results are presented as mean ± standard deviation.

9.8.2 INHIBITION OF THE COLLAGENASE ENZYME WITH SYNTHETIC SUBSTRATE

The alkaloid extracts of *C. angustum*, *P. illyricum* (bulbs and leaves) and *L. nicaeense* were tested on Collagenase assays that uses FALGPA as substrate.

The *Clostridium hystolyticum* collagenase (EC.3.4.23.3) assay was based on the assay described by van Wart et al. [70], slightly modified for use with a 96-well microtiter plate: enzyme was dissolved in 50 mM tricine buffer (with 10 mM $CaCl_2$ and 400 mM NaCl), pH 7.5, to furnish 0.8 units/mL (according to the supplier's activity data); substrate FALGPA was prepared 2 mM in that same buffer. An amount of 25 µL buffer, 25 µL H_2O or inhibitor, and 25 µL enzyme was loaded each well of the 96-well microtiter plate, and after 15 min of preincubation, 50 µL of substrate was added. Absorbance was measured at 340 nm immediately and at 2-min intervals for 20 min. Enzyme activity was estimated by linear regression of the absorbance values recorded during that time. For comparative purposes we also assayed EGCG (epigallocatechin gallate) as a known inhibitor.

The percentage inhibition of enzyme activity was calculated by the following formula:

$$\%\text{inhibition} = [1- (\Delta\text{Abs/min}_{\text{sample}} / \Delta\text{Abs/min}_{\text{negative control}}) \times 100]$$

IC 50 (concentration necessary for 50% inhibition of enzyme activity) was calculated by constructing a linear regression curve. A Lineweaver-Burk (L-B) plot was constructed to calculate the kinetic parameters (K_m expressed in mM and V_{max} in nmol/s) of the enzymatic reaction without and with *P. illyricum* bulbs extract, at the IC_{50} concentration.

Four substrate concentrations in the range of 0.5 mM-2.5 mM were assayed to elucidate the mechanism of inhibition by the bulbs extract of *P. illyricum*.

9.8.3 INHIBITION OF THE COLLAGENASE ENZYME WITH COLLAGEN SUBSTRATE

Plants extract of *C. angustum*, *P. illyricum* (bulbs and leaves), *L. nicaeense*, and three pure compound, lycorine, vittatine and hordenine, respectively at concentrations of 333 µg/mL for extracts and 0.4 mM for pure compounds, were tested with this assay, where the amount of hydroxyproline (4-hyp) which is produced is directly proportional to the amount of the degraded collagen. Method of Mandrone et al. [71] foresees: Collagen from bovine tendon (2 mg) was placed in 1.5 mL test tubes with 100 µL of tested compounds or water (negative control) and 100 µL of tricine buffer 50 mM. After 1h of incubation, 50 µL of a 2mg/mL collagenase stock solution (from *Clostridium hystolyticum*) was added to the mixture to obtain a final volume of 300 µL. The samples were incubated at 37°C (in stove) for 24h. After the request incubation time, samples were vigorously vortexed and centrifuged for 10 min at 12000 rpm. This procedure allows to obtained the non-hydrolyzed collagen packed in the bottom of the tube, thus 150 µL of supernatant were transferred in another test tube and totally volume evaporated in Speed-wac at 80°C for 1h. This step is necessary in order to dried and concentrate the samples moreover. The high temperature allow to a complete H-Pro release from small amino acids sequences derived from the hydrolysis of the starting collagen. Then, 50 µL of deionized water were added to each sample and vortexed. Finally, 100 µL of chloramine T were added and allows to react for 20 min, after that 50 µL of Earlich reactive were added and simples were incubated for 5 min in a 56 °C bath.

A calibration curve was constructed using 50 µL of standard H-Pro at different concentration (from 0 to 50 µM) treated in same conditions of the samples. As a reference compound was used EGCG.

Absorbance was recorder in microplate reader at 557 nm and the 4-hyp concentration was calculated by interpolation in the calibration curve.

% Inhibition of collagen degradation was obtained by the following formula:

$$\% \text{ inhib Coll. Degr.} = [1- ([4\text{-hyp}]_{sample}/ [4\text{-hyp}]_{Neg\ ctrl})] \text{ x}100$$

9.8.4 INHIBITION OF THE TYROSINASE (PPO) ENZYME

Plants extract of *C. angustum*, *P. illyricum* (bulbs and leaves), *L. nicaeense*, and three pure compound, lycorine, galanthamine and hordenine, were tested with Tyrosinase assay. The tyrosinase inhibition assay was performed by Venditti et al. (2013). In the Mushroom tyrosinase (EC 1.14.18.1), nine units of enzyme (1 unit being defined by the producer as the ΔA 280 of 0.001 min^{-1} at pH 6.5 at 25 °C in 3 mL of reaction mix containing L-tyrosine) and plants extract at concentrations of 250 µg/mL were incubated for 5 min in 0.1 M sodium phosphate buffer, pH 6.8, in 0.1 mL final volume. The specific activity of the enzyme is 1715 U/mg. L-DOPA (final concentration = 3 mM) was added to start the reaction, and the formation of dopachrome was immediately monitored for 5 min at 490 nm in a microplate reader under a constant temperature of 30 °C. ΔAbs values were calculated in the first 240 s and referred to 1 min. The percentage inhibition of enzyme activity was calculated by the following formula:

$$\%\text{inhibition} = [1- (\Delta \text{Abs/min sample} / \Delta \text{Abs/min negative control}) \times 100]$$

A negative control was obtained by adding water, instead a positive control was performed using kojic acid, a wellknown tyrosinase inhibitor.

9.9 ANTIMICROBIALS ASSAYS

9.9.1 ANTIBACTERIAL AND ANTIFUNGAL ASSAY ON ATCC STRAINS

The following ATCC (American Type Culture Collection) strains were used: *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Streptococcus pyogenes* (ATCC 19615), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 9591), *Proteus mirabilis* (ATCC 9921), *Candida albicans* (ATCC 10231), *C. tropicalis* (ATCC 13803), *C. parapsilosis* (ATCC 22019), *C. glabrata* (ATCC 15126).

In vitro antimicrobial activity was determined by a broth micro-dilution method using 96-well plates and according to National Committee for Clinical Laboratory Standards (NCCLS)-recommended procedures. Mueller-Hinton (MH) broth (Bio-Rad) was used for all microbial strains, except for S. pyogenes which was grown in MH broth supplemented with 3% lysed horse blood (Bio-Rad).

Microbial suspensions at 5 × 105 CFU/mL were incubated with 2-fold serial dilutions starting from 2.5 mg/mL of extract sample (at least 6 dilutions). The subsequently controls were included in all experiments: extract-free growth control, bacterial inoculum tested with 250 µg/mL of gentamicin (Sigma-Aldrich) or fungal inoculum with 8 µg/mL Amphotericin (Sigma-Aldrich) (positive control), 2-fold serial dilutions of the tested extract inoculated with blank medium without the inoculums, measuring both the background turbidity and the sterility of all procedures (negative control), and 2-fold serial dilutions of DMSO (starting from 6.25% in broth medium) inoculated with the microbial suspension assessing the interference effect of the solvent (solvent control). The microplates were incubated at 37°C for 8-24 h (and 48 h for Candida albicans) and microbial growth was estimated as optical density (OD at 630 nm) determined by the Multiskan Ascent (Thermo Scientific) microplate reader. Results were evaluated as follow: for each sample the net OD value was calculated (ODsample - ODnegative control), then the concentration required to produce 50% growth inhibition (IC_{50} value) was determined, relative to the solvent control. The extracts was assayed in quadruplicate and repeated in three different days, for each microorganism.

9.9.2 ANTIBACTERIAL AND ANTIFUNGAL ASSAY ON CLINICAL ISOLATES

The same analytical procedure used for the ATCC strains was used for clinical isolates. The multidrug resistant (MDR) *Klebsiella pneumoniae* clinical isolates (resistant to β-lactams, cephalosporins, fluoroquinolones, aminoglycosides, macrolides, tetracyclines and carbapenems), the MRSA, and *Candida albicans* clinical isolates were from patients hospitalized in the St. Orsola-Malpighi University Hospital in Bologna, Italy. Routine biochemical identification and antimicrobial susceptibility testing were carried out using the Vitek2 semi-automated system (BioMérieux, France).

9.10 DEVELOPMENTS OF IN VITRO PLANT CELL CULTURES

9.10.1 SELECTION OF MEDIA FOR IN VITRO DEVELOPMENT

For in vitro development of *C. angustum* and *P.illyricum* plants were tested three different media, indicatively called as: MS8 (callus medium), MSL (organogenetic medium), and MSC (shoot formation medium).

- MS8: MS medium (Murashige and Skoog) [72], supplemented with 2,4-dichlorophenoxyacetic acid (0.5 mg/L), kinetin (0.2 mg/L), 3% sucrose and 0.3% Phytagel.
- MSL: MS medium, supplemented with 1-Naphthaleneacetic acid (1.15 mg/L), 6-Benzylaminopurine (2 mg/L), 3% sucrose and 0.3% Phytagel.
- MSC: MS medium, supplemented with 2,4-dichlorophenoxyacetic acid (4mg/L), 6-Benzylaminopurine (2mg/L), 3% sucrose and 0,3% Phytagel.

For micropropagated plants of *L. nicaeense*, developed by the Center for Research in Agriculture (CRA) in Sanremo, the medium used for propagations consisted in: MS medium (Murashige and Skoog) supplemented with Indol butyric acid (0.5 mg/L), 3% sucrose, and 0.3% phytagel; called by us for convenience IBA medium.

9.10.2 EXPLANTS FROM IMMATURE FRUITS

For *in vitro* cell cultures development, immature fruits of *C. angustum* and *P. illyricum* were selected.

Fruits were excised, disinfected with 70% ethanol for 3 min, followed by sodium hypochloride solution (20% commercial bleach, v/v) for 10 min, and then rinsed with sterile distilled water 6–7 times. The disinfected explants were aseptically transferred to Petri dishes containing MS medium called MS8 (MS medium supplemented with 2,4-dichlorophenoxyacetic acid (0.5 mg/L), kinetin (0.2 mg/L), 3% sucrose and 0.3% Phytagel).

Cultures were kept in the growth chamber at 22 °C under 16h light and 8h dark conditions (white fluorescent light, intensity 50 $\mu mol\ m^2\ s^{-1}$).

CHAPTER 10

RESULTS AND DISCUSSION

10.1 QUALITATIVE ANALYSIS OF THE EXTRACTS BY GC-MS

The alkaloid extracts obtained separately from *C. angustum*, *P. illyricum*, and *L. nicaeense* plants, were analysed by GC-MS, in order to obtain their qualitative analysis. In tables **10.1**, **10.2**, and **10.3**, are reported detected alkaloids for each extract, with their retention time, molecular ion, and their expression in terms of TIC % (Total Ion Current). In all extracts have been identified different types of alkaloids, all previously known except for leaves extract of *P. illyricum*, where has been identified a compound with a spectrum fragmentation of alkaloid type and molecular ion of 389 $[M]^+$, but not recognizable with available database.

These plants have never been undergone at phytochemical screening; thus, it was possible to identify for each extract important alkaloids, like: galanthindole in *C. angustum*, a new alkaloid with non-fused indole ring [73], hordenine in *L. nicaeense*, a protoalkaloid, which is a side-reaction in the biosynthetic pathway of the Amaryllidaceae alkaloids, particularly present *in vitro* plant cultures [74], and new, but not yet known alkaloid with molecular ion 389 $[M]^+$, from leaves extract of *P. illyricum*.

10.2 QUANTITATIVE ANALYSIS OF THE EXTRACTS BY HPLC-DAD

In tables **10.4, 10.5,** and **10.6** are shown the amounts of alkaloids detected with HPLC-DAD analysis of plant extracts. By liquid-liquid extraction, aimed at achieving only the alkaloid fraction of crude extracts of *C. angustum*, *P. illyricum* and *L. nicaeense*, were obtained the following extraction yields: 0.09 w/w % for *C. angustum*; 0.43 w/w % from bulbs extract of *P. illyricum*; 0.49 w/w % from leaves extract of *P. illyricum*. As regards *L. nicaeense* were obtained: 0.018 w/w % and 0.023 w/w % respectively for *in vitro* plants (Clone 1) and acclimated plants (Clone 1); 0.009 w/w % and 0.018 w/w % respectively for *in vitro* plants (Clone 2) and acclimated plants (Clone 2); 0.030 w/w % and 0.016 w/w % respectively for *in vitro* plants (Clone 4) and acclimated plants (Clone 4). The amount detected in the extracts follows the % of TIC expressed by qualitative analysis. An important point regards the different extracts of *L. nicaeense*. In fact, the amount of hordenine and anhydrolycorine changes depending on whether are evaluated the extracts from *in vitro* plants or the extracts from acclimated plants. In the specific

case, hordenine is the principal alkaloid in the extracts from *in vitro* plants, while in the acclimated plant extracts the principal alkaloid is the anhydrolycorine. Furthermore, if *in vitro* plant extracts, galanthamine there is only in trace, in acclimated plant extracts is possible to quantify.

According to reports by Berkov *et al*. (2010), hordenine is a protoalkaloid, and represents a predominant alkaloid in undifferentiated calli. Passing from plant grown in vitro to that grown in nature, the amount of protoalkaloids decreases in favor of the alkaloids, until reach the production of more complex alkaloids only in highly differentiated plant tissues, that is, leaves and bulbs.

In the present case have been treated micropropagated plants, some of which have been maintained *in vitro* and other acclimatized in the greenhouse. In both cases these plants derived from *in vitro* cultures, and this probably justifies the low difference in the alkaloid amounts in extracts of *L. nicaeense*. Regarding clones analyzed (Clone 1, 2, 4), there they are not significant differences in the total content of alkaloids.

For this reason was chosen of the total six samples of *L. nicaeense* only one sample (*in vitro* plant of Clone 4), to be subjected to subsequent pharmacological activity assays.

This sample was the one to give greater extraction yield; when in this work will be referenced to the extract of *L. nicaeense* will be considered the extract obtained from *in vitro* plants of the Clone 4.

Table 10.1 Qualitative analysis by GC-MS of *C. angustum* extract. Values are expressed as % of TIC (Total Ion Current)

Alkaloid	Rt	$[M]^+$	Whole plant Extract
Ismine	19,62	257	7,26
Trisphaeridine	19,59	223	1,24
5,6-Dihydrobicolorine	20,56	238	4,11
Galanthamine	21,56	286	5,19
N-Demethylgalanthamine	22,25	272	28,14
Vittatine	22,81	271	1,28
Galanthindole	23,19	281	30,18
Crinamine	25,38	272	1,30
Lycorine	26,81	276	20,98
N-Formylnorgalanthamine	27,54	301	5,03
Macronine	27,90	299	18,01

Table 10.2 Qualitative analysis by GC-MS in the *P. illyricum* extracts. Values are expressed as % of TIC (Total Ion Current)

Alkaloid	Rt	$[M]^+$	Bulbs	Leaves
Galanthamine	21,61	287	7,56	Tr
Sanguinine	21,88	273	6,54	0,92
Vittatine	22,91	271	10,49	17,59
Habranthine	23,78	303	3,05	
Lycorine	26,96	276	24,35	38,86
Leucotamine	28,76	359		13,69
O-Methylleucotamine	28,88	373		20,95
2-Hydroxyhomolycorine	29,83	125	49,3	0,68
11α-hydroxy-*O*-methylleucotamine	30,75	389		3,36

Table 10.3 Qualitative analysis by GC-MS of *L.nicaeense* extracts. Values are expressed as % of TIC (Total Ion Current)

Alkaloid	Rt	$[M]^+$	Vitro Clone 1	Acclimated Clone 1	Vitro Clone 2	Acclimated Clone 2	Vitro Clone 4	Acclimated Clone 4
Hordenine	6,47	165	70,49	40,32	71,56	40,11	70,71	40,77
Galanthamine	21,61	287	tr	5,89	tr	7,34	tr	tr
Anhydrolycorine	23,90	251	28,22	54,20	27,94	51,25	28,78	51,97

Table 10.4 Quantitative analysis in *C. angustum* extract byHPLC-DAD.
Values are expressed as mg of compound / g of FW
For each extract value represents the mean ± standard deviation of three independent determinations by HPLC analysis

Alkaloid	mg/g FW
Ismine	6,26 ± 1,2
Trisphaeridine	1,74 ± 0,2
5,6-Dihydrobicolorine	5,78 ± 0,9
Galanthamine	5,61 ± 0,7
N-Demethylgalanthamine	12,43 ± 1,1
Vittatine	8,39 ± 0,9
Galanthindole	32,13 ± 1,3
Crinamine	1,83 ± 0,05
Lycorine	20,09 ± 0,6
N-Formylnorgalanthamine	6,04 ± 0,7
Macronine	2,09 ± 0,3

Table 10.5 Quantitative analysis in *P. illyricum* extract by HPLC-DAD. Values are expressed as mg of compound / g of FW

Alkaloid	Bulbs mg/g FW	Leaves mg/g FW
Galanthamine	30,80 ± 1,1	Tr
Sanguinine	25,90 ±0,7	4,40 ± 0,9
Vittatine	39,69 ± 1,4	83,30 ± 1,1
Habranthine	14,45 ± 0,9	Tr
Lycorine	105,29 ± 1,2	184,00 ± 1,1
Leucotamine		64,80 ± 1,2
O-Methylleucotamine		99,50 ± 1,4
2-Hydroxyhomolycorine	222,88 ± 1,7	3,20 ± 1,4
11α-hydroxy-*O*-methylleucotamine		15,90 ± 1,2

Table 10.6 Quantitative analysis in *L. nicaeense* extract by HPLC-DAD.
Values are expressed as mg of compound/g of FW

Alkaloid	Vitro Clone 1 mg/g FW	Acclimated Clone 1 mg/g FW	Vitro Clone 2 mg/g FW	Acclimated Clone 2 mg/g FW	Vitro Clone 4 mg/g FW	Acclimated Clone 4 mg/g FW
Hordenine	15 ± 1,1	9 ± 0,5	7 ± 0,5	4 ± 0,3	20 ± 0,4	3 ± 0,7
Galanthamine	tr	4 ± 0,9	Tr	5 ± 0,7	tr	5 ± 0,6
Anhydrolycorine	3 ± 0,3	11 ± 0,2	2 ± 0,2	9 ± 0,2	10 ± 0,1	8 ± 0,3

10.3 ONE AND TWO-DIMENSIONAL NMR CHARACTERIZATION OF THE NEW ALKALOID 11α-HYDROXY-*O*-METHYLLEUCOTAMINE BY *P. ILLYRICUM* EXTRACT

The results of GC-MS analysis of the leaves extract of *P. illyricum* has detected the presence of a compound with spectrum fragmentation of alkaloid type but not identifiable by database. The identification of this compound and the structural elucidation were attained through the combined use of GC–MS, HRMS, and one and two-dimensional NMR techniques. The HRMS of the unknown compound suggested a molecular formula $C_{21}H_{28}NO_6$ for $[M + H]^+$ with a parent ion at m/z 390.1922 (calc. 390.1911). The EIMS showed a molecular ion $[M]^+$ at m/z 389 (15.0%) with a base peak at m/z 286. This value evidenced the loss of the butyryl group at C-3, in accordance with the mass fragmentation of other alkaloids with the same kind of substituent group [75]. The IR spectrum showed absorption of hydroxyl (3429 cm^{-1}), ester (1725 cm^{-1}), and olefin (1624 cm^{-1}) groups. The ^1H NMR of the new compound was very similar to that of *O*-methylleucotamine, isolated for the first time from *Leucojum aestivum* leaves [76], with some differences in the chemical shifts of H-11ß, H-6ß and H-1. A comparison of the chemical shifts of the proton in position H-11ß of *O*-methylleucotamine and the new molecule revealed that H-11ß is more deshielded in the latter (*d* 3.46) than in the former (*d* 2.14). This may be due to the presence, in the new compound, of an interaction with a more electronegative group, which, on the contrary, was not present in *O*-methylleucotamine. The difference between two compounds in terms of molecular weight suggests the presence of a hydroxyl group. The α-position for the hydroxyl substituent is assigned by the small coupling constant J (11ß,12ß) = 4.7 Hz, which suggests a cis relationship between H-11ß and H-12ß and, consequently, the α-position for the hydroxyl substituent. The elucidation of 2D NMR (^1H-^1H COSY, NOESY and HMBC) spectra confirmed this hypothesis and allowed to identify the key for the exact identification of the new molecule. By data obtained with the nuclear magnetic resonance techniques, has been classified new compound as 11α-hydroxy-O-methylleucotamine. In figure **10.1** is shown the structure of the new compound. ^1H NMR spectrum of the 11α-hydroxy-*O*-methylleucotamine is reported in figure **10.2**. The complete assignment of NMR data for 11α-hydroxy-O-methylleucotamine is presented in table **10.7**.

Fig. 10.1 Structure of the new compound 11α-hydroxy-O-methylleucotamine isolated from leaves extract of *P. illyricum* L.

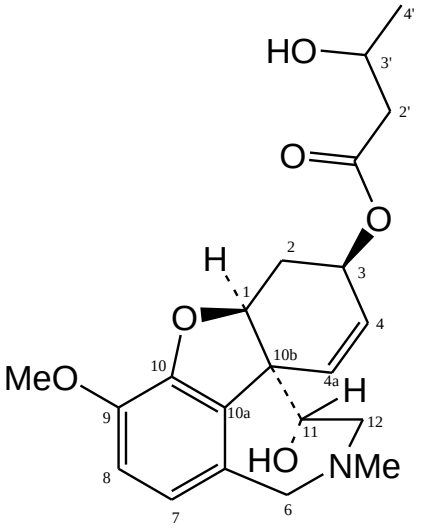


Fig. 10.2 ^{1}H NMR spectrum of 11α-hydroxy-*O*-methylleucotamine

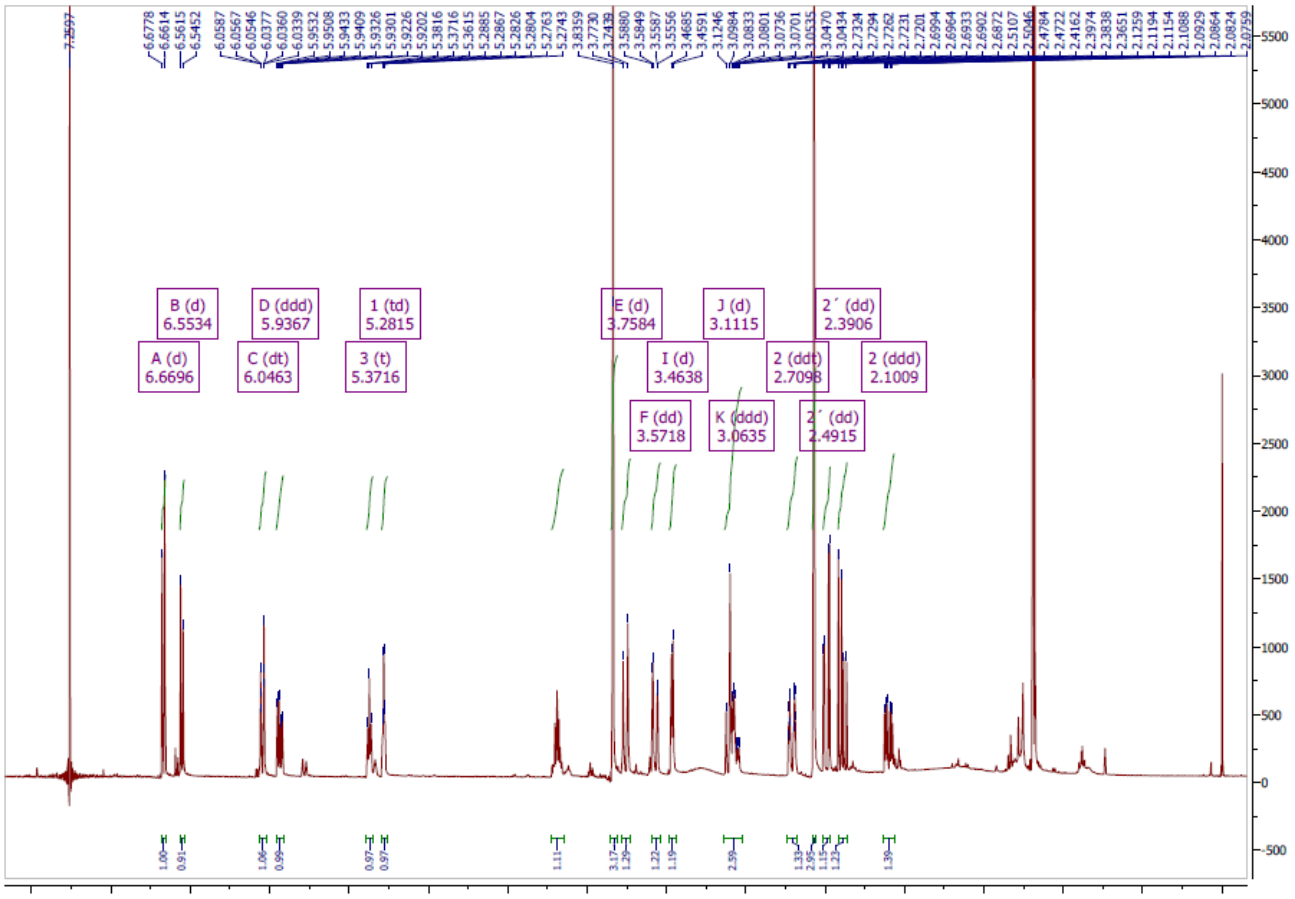


Table 10.7 ^{1}H-NMR, ^{13}C-NMR, COSY, NOESY and HMBC data of 11α-hydroxy-*O*-methylleucotamine

Position	^{1}H δ (J in Hz)	COSY	NOESY	^{13}C δ	HMBC
1	*5.28 td (3.1, 1.0)*	H-2α, H-2β, H-4a, H-4	H-2α, H-2β, H-11β	81.1	C-3, C-4a, C-10b, C-11
2α	2.10 *ddd* (16.5, 5.3, 3.2)	H-1, H-2β, H-3	H-1, H-2β, H-3, H-11β	27.4	
2β	2.71 *ddt* (16.5, 3.1, 1.5)	H-1, H-2α, H-3, H-4	H-1, H-2α, H-3		C-1, C-3, C-4, C-10b
3	5.37 *br t* (5.0)	H-2α, H-2β, H-4, H-4°	H-2α, H-2β, H-4	63.3	C-1, C-2, C-4, C-4a, CO
4	5.94 *ddd* (10.3, 5.0, 1.2)	H-2β, H-3, H-4a, H-1	H-3, H-4a	124.8	C-2, C-3, C-10b
4a	6.05 *dt* (10.3, 1.0)	H-1, H-3, H-4	H-4, H-6β, H-12β	130.1	C-1, C-3, C-4, C-10a, C-10b
6α	*3.57 dd (14.6, 1.5)*	H-6β, H-12α	H-6β, H-7, NMe	63.0	C-7, C-10a, C-12, NMe
6β	3.76 *d* (14.6)	H-6α, H-7	H-4a, H-6α H-12β, NMe		C-6a, C-7, C-10a, C-12
6°				147.9	
7	6.55 *d* (8.2)	H-6α, H-6β, H-8	H-6α, H-8	120.6	C-6, C-6a, C-9, C-10°
8	6.67 *d* (8.2)	H-6β, H-7, OMe	H-7, OMe	111.8	C-6a, C-7, C-9, C-10
9				144.2	
10				128.7	
10a				128.2	
10b				54.0	
11β	3.46 *d* (4.7)	H-12β	H-1, H-2α, H-12β	67.7	C-1, C-10a, C-10b
12α	3.06 *ddd* (13.3, 4.9, 1.6)	H-6α, H-11β, H-12β	H-11β, NMe	61.2	C-6, C-10b, C-11 NMe
12β	3.11 *d* (13.1)	H-12α	H-4a, H-6β, H-11β, NMe		
OMe	3.84 *s*	H-8	H-8, H4´	56.1	C-9
NMe	2.57 *s*		H-6α, H-6β, H-12α, H-12β	49.5	C-6, C-12
CO				172.1	
2'a	2.49 *dd* (16.2, 3.1)	H-2´b, H-3'	H-2´b, H-3', H-4'	43.9	C-3', C-4', CO
2'b	2.39 *dd* (16.2, 9.4)	H-2´a, H-3'	H-2´a, H-3', H-4'		
3'	4.19 *dqd* (9.5, 6.3, 3.1)	H-2'a, H-2'b, H-4'	H-2'a, H-2'b, H-4'	64.1	
4' (3H)	1.19 *d* (6.3)	H-3'	H-2', H-3', OMe	22.7	C-2', C-3', CO

10.4 CYTOTOXICITY

The cytotoxicity assays were carried out on alkaloid extracts of *C. angustum*, *P. illyricum* and *L. nicaeense* for 24h. After exposure of human embryo lung fibroblast with the extracts, was observed only a moderate decrease in cell viability, considering a wide range of concentrations: from 0.625 mg/mL to 0.078 mg/mL.

Initially was considered the reduction of viability due to the solvent used, in this case DMSO. Considering the lack of cell viability inhibition given from DMSO, as a positive control were selected untreated cells. Figure **10.3**, **10.4**, **10.5**, respectively show % of vitality of each extract compared to positive control and solvent

For each extract was calculated the relative concentration (compared to control considered to 100% of vitality) that reduces to 20% cell viability. Is important to consider that the concentrations used of three extracts in the assays reported in this work, were been always below the cytotoxic concentrations.

Concentration that reduces to 20% cell viability for each extract: For *C. angustum*, *P. illyricum* and *L. nicaeense* extracts, values of cell vitality inhibition are greater than 0.625 mg/mL.

Fig. 10.3 Cell viability of *C. angustum* extract.
The graph shows the optical density (OD) that represent the vitality of cells, after cell exposure with increasing concentrations of *C. angustum* extract for 24h compared to control cells.

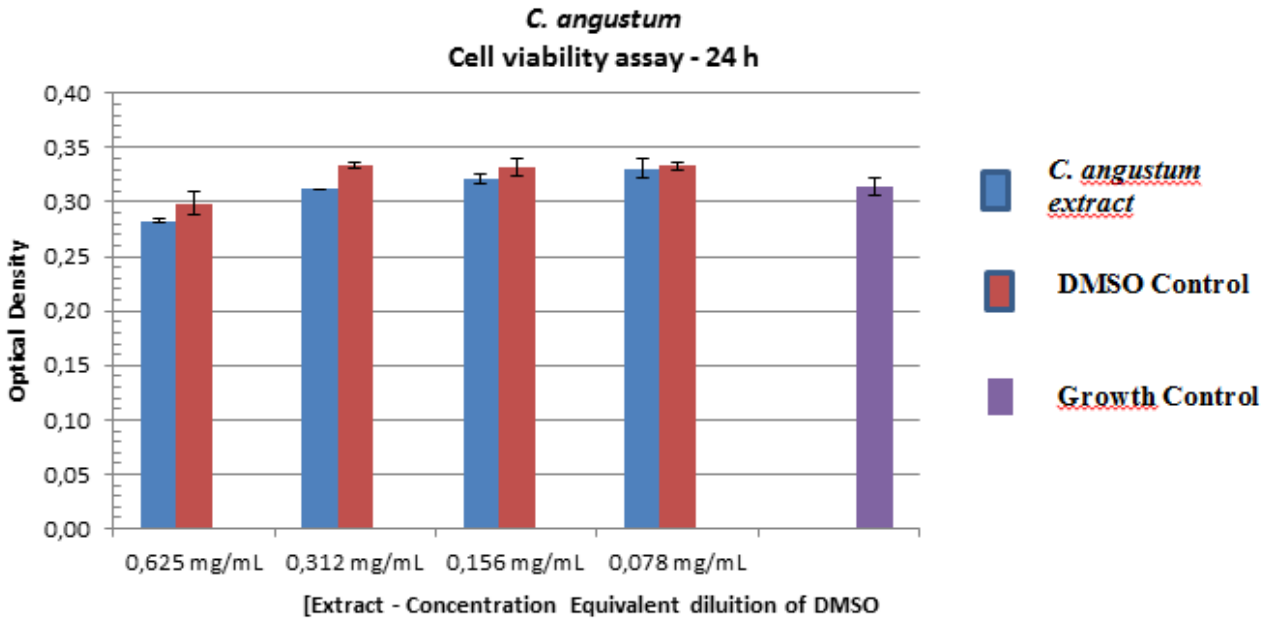


Fig. 10.4 Cell viability of bulbs and leaves extract of *P. illyricum*.
The graph shows the optical density (OD) that represent the vitality of cells, after cell exposure with increasing concentrations of bulbs and leaves *P. illyricum* extract for 24h compared to control cells.

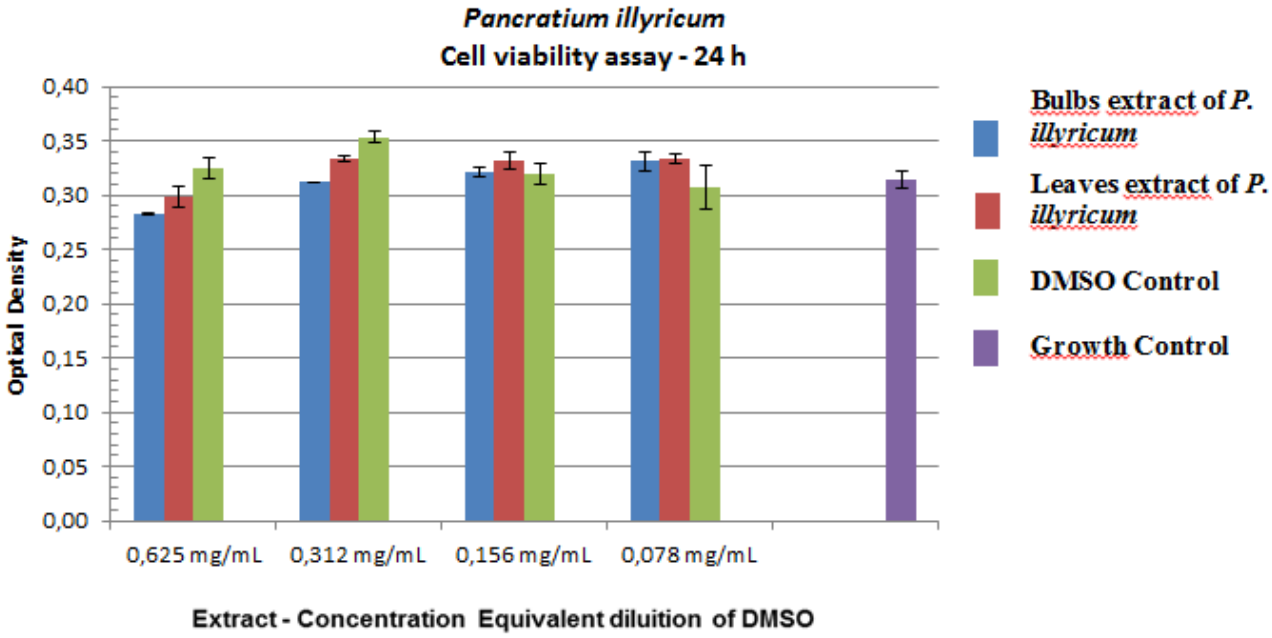
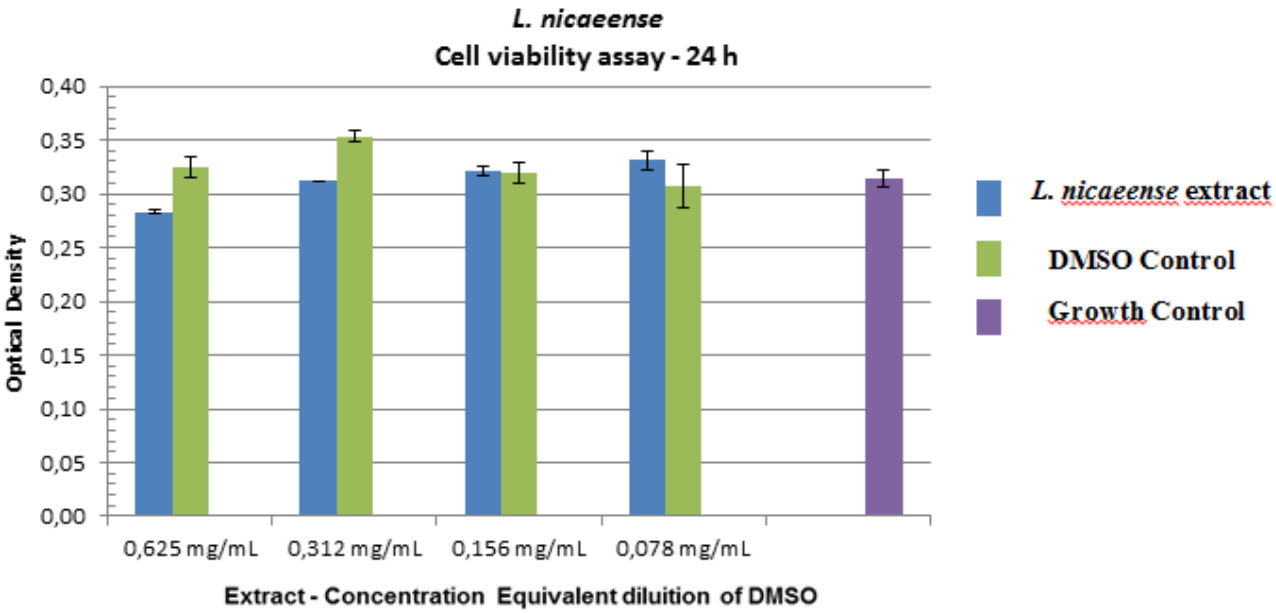


Fig. 10.5 Cell viability of *L. nicaeense* extract.
The graph shows the optical density (OD) that represent the vitality of cells, after cell exposure with increasing concentrations of *L. nicaeense* extract for 24h compared to control cells.



10.5 ANTIOXIDANT ACTIVITY

The alkaloid extracts of *C. angustum*, *P. illyricum* and *L. nicaeense*, were tested with different antioxidant assays in order to assess the potential multiple action to prevent the formation and to remove reactive species.

All three alkaloid extracts have not shown activity against antioxidant assays that involved radical species derived from 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ABTS and 2,2-diphenyl-1-picrylhydrazyl (DPPH). This occurs because the main method of shutting down the radical in this assay is given by transferring of a hydrogen atom or an electron on radical. The examined extracts probably possess stable molecules that do not provide elements capable to altering the radical formed in the assay.

Regarding the β-Carotene bleaching assay, a common method for evaluating the inhibition of lipid peroxidation, was shown again the low ability of the extracts to act as a scavenger. However, while in the ABTS and DPPH assays was not possible to obtain a measurable result, here was possible to carry out a measurement and could be identified in the extract of *C. angustum*, with an IC_{50} of 31.54 µg/mL, a higher

protection activity than other analyzed extracts. In table **10.8** are reported IC_{50} values and the relative TEAC values of the extracts for β-Carotene bleaching assay. Considering that also in this assay is basically implicated the mechanism of donation of a hydrogen, higher reactivity compared to the ABTS and DPPH assay may be due to the fact that in a lipid emulsion, reaction environment of this assay, the hydrophobic repulsion phenomena favor the activity of apolar compounds against the polar ones [77].

Table 10.8 Antioxidant activity of *C. angustum*, *P. illyricum* and *L. nicaeense* extracts by β-Carotene Bleaching test. Data are expressed as IC_{50} values (µg/mL) for extracts and also in µM for the reference compound (Trolox). For a direct comparison, values are expressed also in TEAC (Trolox Equivalent Antioxidant Capacity). Results are means ±SD of three independent experiments with three replicates.

Alkaloid Extract	IC_{50}	TEAC
C. angustum	31,54 ± 0,1	0,11 ± 0,01
***P. illyricum* bulbs**	66,05 ± 0,3	0,052 ± 0,3
***P. illyricum* leaves**	81,47 ± 0,6	0,043 ± 0,6
L. nicaeense	105,9 ± 1,2	0,32 ± 0,001
Trolox (Std)	13,91 µM (3,48 µg/mL) ± 0,1	1

10.6 ENZYMATIC INHIBITION ACTIVITY

10.6.1 ACETYLCHOLINESTERASE INHIBITION

Is well known that the Amaryllidaceae alkaloids are an excellent sources of molecules with inhibitory activity against the Acetylcholinesterase enzyme. The extracts of *C. angustum*, *P. illyricum* and *L. nicaeense* were tested. In addition, considering that the new compound isolated from the leaves extract of *Pancratium illyricum* is galanthamine type, has been tested in order to carry out a direct comparison with the reference standard galanthamine hydrobromide.

The extracts of *C. angustum* and *L. nicaeense* showed no activity, probably because the alkaloids in higher concentrations present in these extracts are not of galanthamine type, and in addition to not having activity prevent the action of the active alkaloids.

The extracts of bulbs and leaves of *P. illyricum*, respectively showed an IC_{50} of 244 ± 1.1 µg/mL and 166 ± 1.7 µg/mL.

Considering the activity of standard and the isolated compound, can see that the activity of the 11α-hydroxy-*O*-methylleucotamine shows no significant difference with that of galanthamine hydrobromide. The IC_{50} for each compounds are: 3.5 ± 1.1 µM for 11α-hydroxy-*O*-methylleucotamine, and 1.5 ± 0.2 µM for galanthamine hydrobromide. In figure **10.6** is shown dose-response curve of the inhibition of AChE enzyme both for reference standard and the new compound. In table **10.9** in order to make a comparison of inhibition capacity of extracts and pure compounds, values are expressed in µg/mL.

In an AChE inhibition screening of several Amaryllidaceae alkaloids from the genus Narcissus, the most active natural alkaloid (ca. 10 times higher than galanthamine) turned out to be sanguinine [78]. This compound showed, compared with galanthamine, a hydroxyl group at C-9 instead of a methoxyl group. An in vitro structure–activity relationship investigation indicates that hydrophilic groups opportunely placed on galanthamine contribute to its effective binding to the enzyme [79]. In the same way, a different spatial orientation of a hydrophilic substituent may increase or decrease interactions within the active site of AChE. For instance, the ß-configuration of the hydroxyl group at position 11 of 11ß-hydroxygalanthamine decreases by 10 times the enzymeactivity compared to its epimer habranthine. 11α-hydroxy-O-methylleucotamine, obtained here from *P. illyricum*, showed a slightly lower activity than 11α-hydroxygalanthamine (IC 50 = 1.61 ± 0.2), probably due to the presence of the

bulky butyryl group at C-3, but it resulted more active than 11ß-hydroxygalanthamine [80]. Thus, theß-configuration of the hydroxyl group at position 11 could make the compound less amenable for interactions with the active site of AChE compared to the butyryl substituent in position 3.

Fig. 10.5 Dose-response curve of the inhibition of AChE enzyme both for galanthamine hydrobromide and 11α-hydroxy-*O*-methylleucotamine.

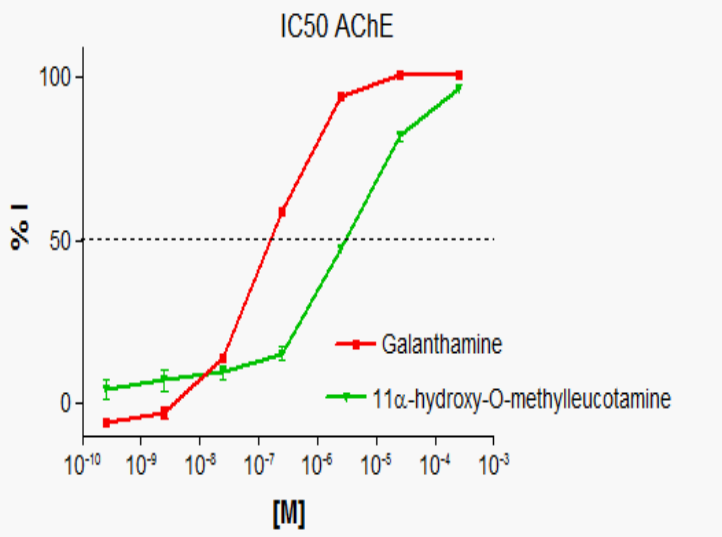


Table 10.9 Values of the inhibition of AChE enzyme for plant extracts and pure compounds expressed in µg/mL.

Alkaloid Extract And Pure Compounds	IC_{50}
P. illyricum bulbs	224 ± 1,1
P. illyricum leaves	166 ±1,7
Galanthamine	0,43 ± 0,2
11α-hydroxy-*O*-methylleucotamine	1,36 ± 1,1

10.6.2 COLLAGENASE INHIBITION

Two different assays were applied as regards the inhibition of collagenase enzyme. The first one involves use of synthetic substrate FALGPA (N-3-(2-Furyl)acryloyl-Leu-Gly-Pro-Ala), and the second one use of a natural substrate such as collagen from bovine tendons. The alkaloid extracts of *C. angustum*, *P. illyricum* and *L. nicaeense* were tested using both methods.

Analyzing the results obtained with the synthetic substrate, extracts have been shown to inhibit the enzyme in so far of: *C. angustum* extract has not inhibitory activity; *P. illyricum* has shown a different inhibitory activity regarding leaves and bulbs extract, in fact, at the same tested concentration of 200 µg/mL, bulbs extract showed 99.61% ± 1.2 of inhibition and leaves extract 47.62% ± 1.6; *L. nicaeense* extract with 200 µg/mL has given an inhibition of 21.76% ± 0.9.

Considering these data the bulbs extract of *P. illyricum* was chosen in order to establish an IC_{50} value and a possible mechanism of action.

In figure **10.6** is shown the diagram of Lineweaver-Burk that demonstrates the mechanism of action of the extract. Variation of V_{MAX} and K_M values compared to the enzyme without inhibitor suggests that the inhibition of the extract on the enzyme is reversible and uncompetitive type. Mechanism probably is attributable to a double action of alkaloid extract; initially chelating Ca^{2+} ions present in the reaction, fundamental for the activation the enzyme, and at the same time probably interacting with the active site of the enzyme. In table **10.10** are listed kinetic parameters on the enzyme inhibition given by the bulbs extract of *P. illyricum*.

The IC_{50} value obtained from the bulbs extract of *P. illyricum* is directly comparable to that of the reference standard epigallocatechin gallate (EGCG) in order of µg/mL.

In table **10.11** is shown IC_{50} value of bulbs extract of *P. illyricum*, and that of the reference compound EGCG.

Is noteworthy that although the bulbs and leaves extract of *P. illyricum* have almost the same qualitative composition, quantitatively alkaloids are found in opposite concentrations. Basically, if an alkaloids prevail in concentration into one extracts, decreases in the other. Moreover considering the belonging alkaloid class, in bulbs extract prevailing alkaloids group is lycorine, whereas in leaves extract there is a balance between alkaloid types. Probably the most important activity of the bulbs

extract than the leaves extract, could be given by the predominance of lycorine-type alkaloids in the former.

In the assay which foresees the use of bovine collagen as a substrate, were tested the same extracts at fixed concentration of 333 µg/mL. Were also tested three pure compounds representing some of the main components in the examined extracts, at fixed concentration of 400 µM. The IC_{50} value of the reference standard (EGCG) in this assay is 360 µg/mL or expressed in molarity 785 µM. Comparing IC_{50} value of epigallocatechin gallate (IC_{50} 4.33 µg/mL and 9.45 µM) obtain with the first method, to achieve the same effect of the first one, is necessary to use concentrations 10 times higher.

By the extracts were obtained the following percentages of inhibition: 39.49% ± 2.0 for *C. angustum* extract, 86.37% ± 1.2 for *P. illyricum* bulbs extract, 83.94% ± 1.9 for *P. illyricum* leaves extract, and 56.96% ± 1.7 for *L. nicaeense* extract. Lycorine, Vittatine and Hordenine, the pure compounds tested, at the analyzed concentration have not shown activity. Table **10.12** shows the percentages of inhibition for extracts and compounds, tested in both assays of collagenase inhibition.

Using this assay, all samples tested showed an activity, except pure compounds. One possible explanation for the apparent discrepancy in the inhibition values can be given by different reaction times and different interaction between enzyme, substrate and inhibitor. In the assay that uses FALGPA as substrate, reaction times is rated in 5 min; in the assay that uses Collagen as substrate, reaction times is rated in 5 hours; in the latter case, has a longer time to develop the inhibitory action or a longer time to neutralize the inhibitor.

In the assay that uses FALGPA as substrate, occurs primarily an interaction between enzyme and inhibitor [81], in the assay with Collagen the interaction is between substrate and inhibitor. Essentially, samples that have no interaction with the enzyme in the first assay may have a greater interaction with the substrate with the second method.

Fig. 10.6 Lineweaver-Burk graphic: mechanism of action of the bulbs extract of *P. illyricum.*

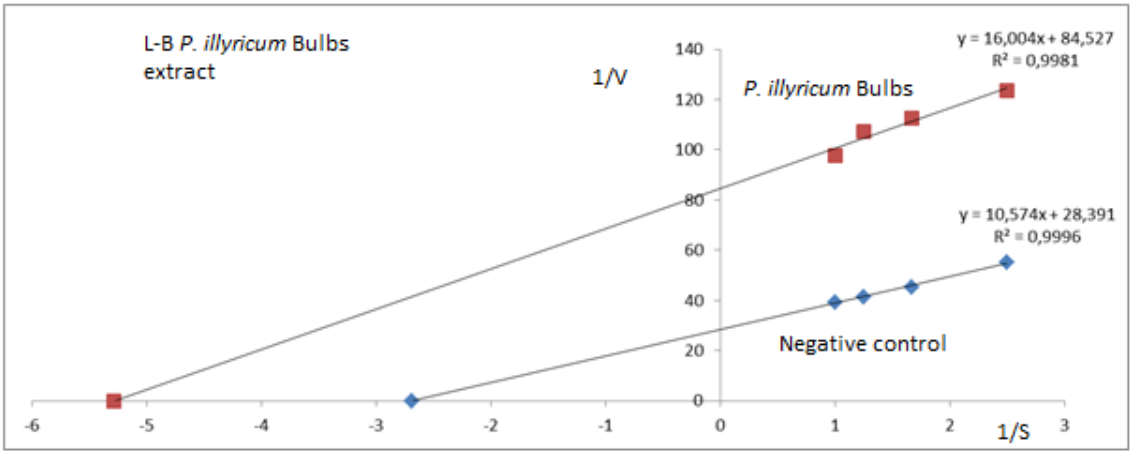


Table 10.10 Kinetic parameters on the enzyme inhibition given by the extract of *P. illyricum*. its IC_{50} value in µg/mL, and that of the reference compound EGCG which is expressed both in uM and as µg/mL.

	Enzyme with Inhibitor	Enzyme without Inhibitor
KM (mM)	0,189	0,372
Vmax (nmol/s)	0,033	0,098

Table 10.11 IC_{50} value in µg/mL of *P. illyricum* bulbs, and that of the reference compound EGCG which is expressed both in uM and as µg/mL.

Alkaloid Extract And Pure Compound	IC_{50} µg/mL	IC_{50} µM
***P. illyricum* bulbs**	25,00 ± 0,1	
EGCG	4,33 ± 0,02	9,45 ±0,5

Table 10.12 Percentage of collagenase inhibition from extracts of *C. angustum*, *P. illyricum* , *L. nicaeense* and pure compounds lycorine, vittatine and hordenine in both collagenase inhibition assays

Alkaloid Extract And Pure Compounds	FALGPA substrate	COLLAGEN substrate
C. angustum	no inhibition	39,49% ± 2,0
P. Illyricum bulbs	99,61% ± 1,2	86,37% ± 1,2
P. Illyricum leaves	47,62% ±1,6	83,94% ± 1,9
L. nicaeense	21,76% ± 0,9	56,96% ± 1,7
Lycorine	-	no inhibition
Vittatine	-	no inhibition
Hordenine	-	no inhibition

10.6.3 TYROSINASE INHIBITION

In preliminary study the extract of *C. angustum*, *P. illyricum*, and *L. nicaeense*, also with three pure alkaloid compounds, like galanthamine, lycorine and hordenine were tested at fixed concentration of 250 µg/mL. Molecule used as a reference was Kojic acid, which has an IC_{50} value of 14.7 ± 2.0 µM (2.09 µg/mL). The percentages of inhibition were: *C. angustum* extract, no inhibition; bulbs extract of *P. illyricum* 34% ± 2.1; leaves extract of *P. illyricum* 10% ± 0.7; *L. nicaeense* extract 32%.± 0.2 Regarding pure compounds: galanthamine no inhibition, lycorine no inhibition, and hordenine 45% ± 0.5 of inhibition.

Is important to note the difference in activity between the bulbs and leaves extracts of *P. illyricum*. The decrease of activity in the leaves extract could be explained by the fact that in this extract tend to predominate compounds as galanthamine and lycorine, that have shown their inactivity by testing separately. In parallel hordenine is one of the major compounds present in the extract of *L. nicaeense*, and is probably the presence of this molecule that confers activity to the extract, in fact tested separately showing a greater inhibitory activity of the extract itself. This inhibition ability of hordenine may be explained in terms of similarity between the dihydroxyphenyl group in L-Dopa and the similar group in hordenine; is also possible that the interaction with non-active components in the extract decreases its function. These data would be in agreement with recent work where hordenine is proposed like compound capable to inhibits melanogenesis in human melanocytes [82].

10.7 ANTIMICROBIAL ACTIVITY

10.7.1 BACTERIAL AND FUNGAL GROWTH INHIBITION ON ATCC STRAINS

Extracts of *C. angustum*, and *P. illyricum*, were tested against ATCC bacterial and fungal strains. *L. nicaeense* extract has not been tested on this kind of activity; as obtained by *in vitro* material, the available amount was not enough to the realization of this assay. The antimicrobial activity of the extracts were evaluated against 9 standard strains of microorganisms, that include *Staphylococcus aureus, S. epidermidis, Streptococcus pyogenes, Enterococcus faecalis, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis* and *Candida albicans*.

The alkaloid extract of *C. angustum* was found to be more active on Gram-positive bacterial strains (*S. aureus, S. epidermidis, S. pyogenes, E. faecalis*) than on Gram-negative ones (*E. coli, K. pneumoniae, P. aeruginosa, P. mirabilis*), with IC_{50} values ranging from 156 to 625 µg/mL after 8 h incubation. Among Gram-negative bacterial strains, an inhibitory activity was observed only on *K. pneumoniae* at 8 h incubation. It is noteworthy to observe that a pronounced activity (IC_{50} 78 µg/mL) against *C. albicans* was found at 24 and 48 h incubation. Based on the chemical structure of the molecules present in the extract and the biological activities reported for these compounds, an hypothesis can be put forward regarding the major candidates responsible for the antimicrobial activity. As regards lycorine, previous reports demonstrated the lack of inhibitory effect of this alkaloid on the growth of *S. aureus, E. coli*, and *P. aeruginosa*, while showing an excellent antifungal activity on *C. albicans* [13] Our results indicate that *Crinum* alkaloid extract did not show any antibacterial activity on *E. coli* and *P. aeruginosa*, showed only a low activity on *S. aureus* (IC_{50} 1.25 mg/ml after 24 h incubation), while having a good antifungal activity on *C. albicans*. This can be explained by the fact that both *S. aureus* and *P. aeruginosa* are able to transform lycorine into its inactive metabolite 2-*O*-demethylungiminorine, instead of the active ungeremine [83]. Since a stimulatory effect on bacterial growth has been reported for 2-*O*-demethylungiminorine, this can explain, in part, the lack of antimicrobial activity of the alkaloid extract on *S. aureus*. The alkaloids of galanthamine-, narciclasine-, tazettine- and haemanthamine-type were also present in *C. angustum* alkaloid extract.

As regards alkaloids belonging to the first three types, no antibacterial or antifungal activities have hitherto been reported, even though a wide array of biological activities have been demonstrated [84]. As regards alkaloids of the haemanthamine-type, represented in this specific case by vittatine and crinamine, an antibacterial activity was demonstrated on *S. aureus* and *C. albicans*. As showed by Evidente et al., (2004), vittatine was reported to be active against these strains (MIC values of 63 and 31 µg/mL, respectively), and an inhibitory activity against *S. aureus* was found for crinamine [85]. Thus, considering the results here obtained on *Crinum* alkaloid extract and data from previous studies on purified alkaloids, it is likely that vittatine and crinamine did play a role in the antimicrobial activity of the extract. Besides these, also galanthindole might contribute to the inhibitory activity. Indeed, the presence of an indole group within this molecule can support this hypothesis, since a key role for this important heterocyclic system in several biologically active molecules exhibiting antimicrobial and antifungal capacity has been reported [86].

It is worth noting that quite often the activity found for the total alkaloid extract is lower than that of pure components, as a consequence of the fact that pure compounds are highly selective in their interaction with the bacterial pathogens [87], thus being attenuated in their potency when mixed with other components in a total extract.

Table **10.13** shows the complete values of *C. angustum* extract at 8h and 24h of incubation with the corresponding reference standard gentamicin.

As regard *P. illyricum* extract, showed no significant activity against bacterial ATCC strains, while it proved particularly active against *Candida albicans*, with IC_{50} values of 39 µg/mL for bulbs extract and 78 µg/mL for leaves extract at 24h of incubation. The extract of bulbs and leaves of *P. illyricum* were also tested with *C. albicans* strain until 48h of incubation, obtaining respectively IC_{50} values of 78 µg/mL and 156 µg/mL. Analyzing the alkaloids found in these extracts, to light of the foregoing, can be attributed the absence of activity against bacterial strains at the preponderant presence in the extracts of the alkaloids lycorine and galanthamine type; the former are transformed into inactive metabolites by *S. aureus*, and the latter do not have antibacterial activity. Rather lycorine-type alkaloids are an excellent antifungal activity, probably for this reason, in bulbs where they are in greater quantity than that in leaves, is shown increased antifungal activity.

Considering the good activity shown on this *Candida* species, is thought to test bulbs and leaves extract of *P. illyricum*, on three different *Candida* species responsible for nosocomial infections.

Thus, have been tested: *C. parapsilosis*, *C. tropicalis*, and *C. glabrata*, at 24-48h of incubation. The IC_{50} values achieved are comparable with those obtained for the *C albicans* at 24h incubation. Regarding the *C. parapsilosis*, bulbs activity is higher than in leaves, but constant in both at 24 and 48h. *C. tropicalis* . resistance is greater than the other *Candida* species, but with constant values both in bulbs and leaves extract. *C. glabrata*, possesses values of inhibition comparable with *C parapsilosis* and *C. albicans* only to 24h. *C. albicans*, shows IC_{50} values comparable to those shown in *C. parapsilosis*; even in this case bulbs possess better inhibitory activity.

In table **10.14** are shown IC_{50} values at 24 and 48 h of incubation, and the relative reference standard Amphotericin, for the four *Candida* species analyzed with bulbs and leaves extract of *P. illyricum*

Table 10.13 Complete values of *C. angustum* extract at 8h and 24h of incubation on microbial ATCC strains with the corresponding reference standard gentamicin.

Microorganism	IC_{50} (8h-incubation) (μg/mL)	IC_{50} (24h-incubation) (μg/mL)	Positive control IC_{50} 24h (μg/mL)
S. aureus	312 ± 1,4	1250 ± 3,4	1.0 ± 0,01
S. epidermidis	156 ± 1,1	625 ± 1,8	2.0 ± 0,01
S. pyogenes	312 ± 1,9	312 ± 1,4	4.0 ± 0,02
E. faecalis	625 ± 2,0	-	4.0 ± 0,02
E. coli	-	-	4.0 ± 0,03
K. pneumoniae	625 ± 2,0	-	4.0 ± 0,02
P. aeruginosa	-	-	8.0 ± 0,02
P. mirabilis	-	-	4.0 ± 0,03
*C. albicans**	no growth	78 ± 1,5(48 h: 78)	2.0 ± 0,02

:- no inhibition. * IC_{50} values for *C. albicans* ATCC 10231 were determined after 48 h incubation and Positive Control was nystatin

Table 10.14 Complete values of *P. illyricum* bulbs and leaves extract at 24h and 48h of incubation on *Candida* ATCC strains with the corresponding reference standard Amphotericin

	Bulbs extract of *P. Illyricum*		Leaves extract of *P. illyricum*		Positive Control Amphotericin IC_{50} (µg/mL)
	IC_{50} 24h (µg/mL)	IC_{50} 48h (µg/mL)	IC_{50} 24h (µg/mL)	IC_{50} 48h (µg/mL)	
C. parapsilosis	39 ± 0,2	39 ± 0,3	78 ± 0,4	78 ± 0,4	2,0 ± 0,01
C. tropicalis	156 ± 0,6	310 ± 1,3	156 ± 0,6	310 ± 1,2	4,0 ± 0,03
C. glabrata	39 ± 0,4	156 ± 0,4	78 ± 0,4	310 ± 1,4	4,0 ± 0,03
C. albicans	39 ± 0,2	78 ± 0,2	78 ± 0,6	156 ± 0,8	4,0 ± 0,02

10.7.2 BACTERIAL AND FUNGAL GROWTH INHIBITION ON CLINICAL ISOLATES

Considering the promising inhibitory activity of the extracts of *C. angustum* and *P. illyricum* against different ATCC strains, both bacterial and fungal type, was carried out a direct analysis on clinical isolates.

As regards the C. angustum, was tested clinical isolates of methicillin-resistant *S. aureus* (MRSA) and carbapenemase-producing *Klebsiella pneumoniae* (KPCs). Were chosen these bacteria, as, the former is the main source of clinically significant infections; the latter is one of the most resistant gram negative bacteria, the only one wherein the *C. angustum* extract showed a good inhibitory activity at 8h of incubation.

Tests were carried out both at 8 and 24h of incubation, and the results showed that: KPCs ones were inhibited by the *C. angustum* extract only at 8h of incubation with a IC_{50} range between 312 and 625 µg/mL, while a bacteriostatic effect was found against the MRSA isolates with IC_{50} values ranging from 156 to 1250 µg/mL.

These data are consistent with those obtained on the ATCC strains, and are not significantly different between them, whereas that patients from which come represent different clinical cases.

Table **10.15** shows the IC_{50} of the *C. angustum* extract at 8 and 24h of incubation on MRSA and KPCS clinical isolates.

As regards *P. illyricum* were tested *C. albicans* clinical isolates, as, this species is a major cause of nosocomial infections. Furthermore, whereas it in ATCC strains the extract to having increased activity was bulbs extract, and considering that this extract contains lycorine, alkaloid which has been given high antifungal activity, as reported in Evidente et al., (2004), on clinical isolates were tested: bulbs extract of *P. illyricum*, Lycorine, and reference standard Amphotericin, considering only 24h of incubation as shown in European guidelines EUCAST (European Committee for susceptibility to antifungal drugs) [88].

Whereas it isolates derived from patients with different clinical histories, there are no meaningful significant differences, except to clinical isolate derived from patient g, where the activity of bulbs extract, lycorine and the reference standard, are more pronounced compared to the other isolated.

By comparing IC_{50} values of bulbs extract of *P. illyricum* and then the purified compound lycorine, higher inhibitory activity is exhibited by lycorina in agreement with reported by Cheesman et al. (2012) In fact, often the activity found for the total alkaloid extract is lower than that of pure components, as a consequence of the fact that pure compounds are highly selective in their interaction with the bacterial pathogens.

By averaging the IC_{50} values obtained to clinical isolated, is possible to establish that for bulbs extract of *P. illyricum* IC_{50} value is 39 µg/mL, while for the purified compound lycorine IC_{50} value is 7 µg/mL.

Table **10.16** shows complete IC_{50} values on the Clinical Isolates of *C. albicans* by *P.illyricum* bulbs extract, lycorine and reference standard Amphotericin.

Table 10.15 IC_{50} values on the Clinical Isolates of *C. angustum* extract.

Microorganism	IC_{50} (8h-incubation) (μg/mL)	IC_{50} (24h-incubation) (μg/mL)
S. aureus, methicillin resistant (MRSA) [a]	625 ± 2,4	625 ± 2,0
S. aureus, methicillin resistant (MRSA) [b]	625 ± 2,2	1250 ± 3,0
S. aureus, methicillin resistant (MRSA) [c]	312 ± 2,2	1250 ± 3,1
S. aureus, methicillin resistant (MRSA) [d]	312 ± 2,1	625 ± 2,2
S. aureus, methicillin resistant (MRSA) [e]	78 ± 1,4	156 ± 1,4
S. aureus, methicillin resistant (MRSA) [f]	312 ± 1,9	625 ± 2,2
S. aureus, methicillin resistant (MRSA) [g]	156 ± 1,3	625 ± 2,0
K. pneumoniae carbapenem resistant (KPC) [1]	312 ± 2,2	-
K. pneumoniae carbapenem resistant (KPC) [2]	625 ± 2,4	-
K. pneumoniae carbapenem resistant (KPC) [3]	-	-

Notes: - : No inhibition; Letters and numbers in superscripts indicate clinical isolates from different patients.

Table 10.16 IC_{50} values on the Clinical Isolates of *C. albicans* by *P.illyricum* bulbs extract, lycorine and reference standard amphotericin. Letters in superscripts indicate clinical isolates from different patients

	Bulbs extract of *P. illyricum* IC_{50} (µg/mL) 24h of incubation	Lycorine IC_{50} (µg/mL) 24h of incubation	Positive Control Amphotericin IC_{50} (µg/mL) 24h of incubation
C. albicans[a]	39± 0,3	7 ± 0,3	0,10 ± 0,001
C. albicans[b]	78 ± 0,3	10 ± 0,4	0,15 ± 0,001
C. albicans[c]	39 ± 0,2	8 ± 0,4	0,10 ± 0,01
C. albicans[d]	10 ± 0,2	4 ± 0,3	0,05 ± 0,01
C. albicans[e]	19 ± 0,2	4 ± 0,2	0,14 ± 0,02
C. albicans[f]	10 ± 0,3	4 ± 0,3	0,03 ± 0,03
C. albicans[g]	0,61 ± 0,01	0,9 ± 0,01	0,03 ± 0,001
C. albicans[i]	3 ± 0,03	0,9 ± 0,01	0,06 ± 0,002
C. albicans[d]	39 ± 0,2	7 ± 0,2	0,08 ± 0,002
C. albicans[l]	39 ± 0,2	7 ± 0,2	0,15 ± 0,001
C. albicans[m]	39 ± 0,1	7 ± 0,3	0,15 ± 0,001

10.8 *IN VITRO* DEVELOPMENT OF PLANT CELL CULTURES FROM *C. ANGUSTUM, P. ILLYRICUM, L. NICAEENSE*

Were realized vegetal explants from immature fruits of *C. angustum* and *P. illyricum* for *in vitro* development of plant cell cultures. As regards *L. nicaeense* have been developed subcultures from micropropagated plants produced in CRA Center of Sanremo.

The explants obtained from *C. angustum* have proved to be completely refractories to the development of callus; by subjecting the explants to different types of culture media, like: culture media for callus development (MS8), for organogenesis development (MSL), and for shoots regeneration (MSC); the explants have never led to the production of *in vitro* cell cultures.

P .illyricum explants, subjected to growth in a medium that favored callus formation, has been quiescent nearly a year, then, has led to the development of very slow-growing calli. The lack of friability in the *P. illyricum* calli, has not allowed development in bioreactors. Transferring calli in an organogenetic culture medium used for *L. nicaeense* (IBA medium), were obtained plants can undergo to in vitro micropropagation.

Figure **10.6** shows some of the calli samples obtained from *P. illyricum*.

Regarding micropropagated plants of *L. nicaeense*, were subjected to cycles of micropropagation until to obtain a quantity that allowed phytochimical and biological analysis.

Figure **10.7** shows a representative sample of micropropagated plants obtained from *L. nicaeense.*

Results obtained as regards *in vitro* development of these plants, are consistent with those reported in the scientific works, in fact, *in vitro* development of plants belonging to the Amaryllidaceae family, occurs always through the slow formation of shoots or regenerated plants, as is shown in Berkov et al. (2010).

Fig. 10.6 Calli samples obtained from *P. illyricum*.

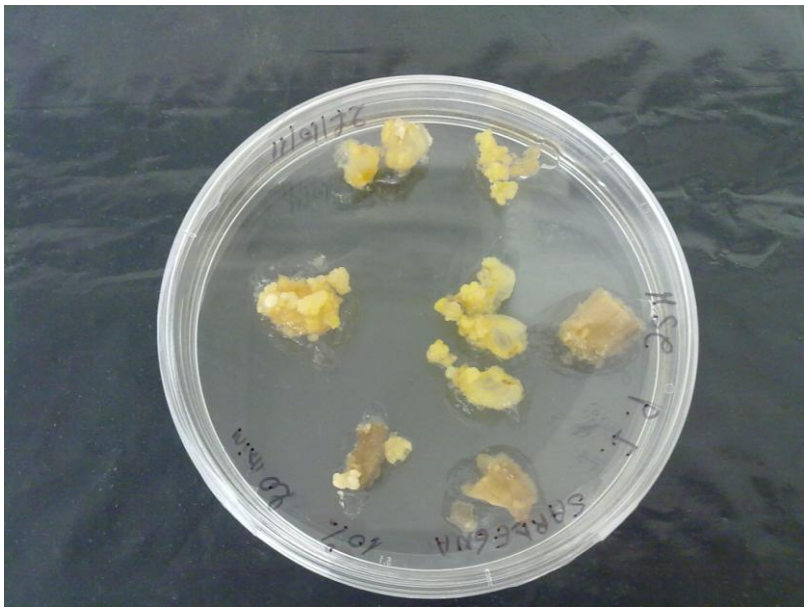


Fig. 10.7 Sample of micropropagated plants obtained from *L. nicaeense*.

CONCLUSIONS

In the present study were analyzed extracts enriched in alkaloids obtained from three different plants of the Amaryllidaceae family. In particular were investigated the plant extracts of: *Crinum angustum*, *Pancratium illyricum*, and *Leucojum nicaeense*.
Is known as the plants, and in the specific, those belonging to this family, are sources of numerous molecules with important pharmacological activities for man. For this reason the extracts obtained were analyzed to phytochemical analysis, with a view to isolate novel compounds with pharmacological property, and were tested on a wide range of biological activities with the aim of identifying the one to which better suited.

Phytochemical analysis has allowed to isolate and characterize a new molecule of galanthamine type from the leaves extract of *P. illyricum*.. This compound, 11α-hydroxy-*O*-methylleucotamine, has been tested in the inhibition assay of the Acetylcholinesterase enzyme (AChE), and showed inhibitory activity equivalent to that of the molecule commercially (galanthamine) for treatment of Alzheimer disease symptoms. 11α-hydroxy-*O*-methylleucotamine could be a new compound to use in symptoms of this disease.

Data derived from the antioxidant assays, highlight that the extracts tested do not possess antioxidant activity. However is important to emphasize the fact that the extracts were undergone in assays to involve transfer of electrons or hydrogen ions, and molecules present in these plant extracts do not possess this capacity. Likely using the extracts of *C. angustum*, *P. illyricum* and *L. nicaeense*, in enzymatic assays, as antioxidants with indirect action, where many factors are involved, not only the role of neutralization relate to reactive oxygen species, or considering the protective role of these extracts on substrates attacked by radical species, these extracts could provide antioxidant activity.

Identify compounds which possess activity against enzymes involved in several diseases could be an important field of study, as, a single compound could be interested in treatment of different pathologies, and probably at the same time treating a disease

could be prevent other. For this reason was decided to test the extracts of *C. angustum*, *P. illyricum* and *L. nicaeense* against collagenase, enzyme belonging to the matrix metalloproteinase family, a class involved in both physiological and pathological processes. Of all matrix metalloproteinase, collagenase, is surely that less specific for certain pathologies; however, at the same time, allows to perform a screening of compounds potentially active in this class of enzymes. Therefore the extracts were tested against the collagenase enzyme with two different assays, where were employed two different substrates. In the first one was used synthetic substrate (FALGPA), while in the second one was used a natural substrate (collagen from bovine tendon). In both assays the most promising extract has been the *P. illyricum* bulbs extract, with an IC_{50} value comparable to that of the positive control, epigallocatechin gallate (EGCG). Through the mechanism of action studied by the first essay, can be assumed for *P. illyricum* bulbs extract a double action on the enzyme; Ca^{2+}chelation in the reaction environment fundamental to functionality of the enzyme, and an interaction with the active site of the enzyme. Even if these are preliminary studies, and much more can be done, both, to study the mechanism of action of this extract, that to study detailed the individual classes of matrix metalloproteinase, the obtained results allow to identify a group of molecules, alkaloids, as possible compounds of interested in the study of this wide and complex class of enzymes. In addition, the preliminary data obtained with the second test have confirmed the strong inhibitory activity of the bulbs extract of *P. illyricum*, has revealed the activity of *L. nicaeense* extract (56.96% of inhibition) not detected by the first assay, and has allowed to discriminate the activity of certain molecules in the extracts, excluding amongst the pure compounds hypothetically responsible for the inhibition, as: lycorine, vittatine and hordenine.

As regards the tyrosinase enzyme, also known as polyphenol oxidase (PPO), preliminary data obtained with enzymatic assay, allows to identify rather than an extract and consequently a plant as a possible candidate for the inhibition of this enzyme, a class of pure compounds. In fact, comparing the activity shown by the extracts and by the purified molecules, that make up a large part of the extracts, it can be concluded that: hordenine has good inhibitory activity, and given the lack of activity of lycorine, the former is what gives activity to the extract of *L. nicaeense*. Considering the lack of activity given by lycorine and galanthamine, and the activity shown by the extracts of *P.*

illyricum (34% bulbs extract and 10% leaves extract), can say that: where prevail compounds lycorine and galanthamine type, there isn't inhibitory activity, as occurs for example in the leaves extract of *P.illyricum* where these two classes of alkaloids are in balance; in bulbs extract of *P. illyricum*, where is present in large amounts a third class of alkaloids, or rather homolycorine type, the extract showed good inhibitory activity. Basically, there are two molecules in the tested extracts, which are good candidates for inhibition of the enzyme tyrosinase: hordenine compound and alkaloids homolycorine type. The importance of finding molecules able to inhibit this enzyme covers different fields, those where the tyrosinase plays a key role: development of melanoma, skin browning, and last but not least the molting in the insects in the agrochemical business.

The activity shown by the extracts of *C. angustum* and *P. illyricum* before on microorganisms ATCC strains and after on clinical isolates multi-drug resistant, nominate some of the alkaloids they contain as potential compounds to treat, alone or in association with compounds multi-drug resistant, infections by pathogens to date harder to eliminate. As regards the extract of *C. angustum* was assumed like component responsible of the activity, the galanthindole. In future studies should be try to isolate this compound and test individually, in order to determine its real activity, and probably as occurs in this type of assays, to compare its activity with that of the total extract. Like occurred in fact in the bulbs extract of *P. illyricum*, the activity shown by the extract is lower than that shown by lycorine, purified alkaloid from *P. illyricum* extract.

As regards the development of *in vitro* cell cultures, these plants of the Amaryllidaceae family, have proved refractoriness to the development of in vitro cultures, or rather, the only *in vitro* development to which lend themselves is that to form regenerated plants from tissues explants. Although this is not the desired result, as, a development of suspension cells would have led to a large quantities and rapid production of secondary metabolites, the ultimate goal to preservation of plant species and the production of alkaloids, was not invalidated. For instance tests conducted on micropropagated plant extracts of *L. nicaeense* have shown that *in vitro* production of natural compounds, can equate that in nature, and can represent an excellent source of pharmacologically active compounds.

By concluding, the extracts obtained from plants of, *C. angustum*, *P. illyricum* and *L. nicaeense*, belonging to the Amaryllidaceae family, have been the source of molecules with high pharmacological potential, whose applicability covers various fields, like: neurodegenerative diseases, antineoplastic activity, anti-aging, and antimicrobial activity. The production of these compounds by means of plant cell cultures allows a higher and selective production, independent of the availability and the seasonality of the plant, not least allows the preservation of plant species.

REFERENCES

[1] J.K. Borchardt. The beginnings of drug therapy: Ancient mesopotamian medicine, Drug News Perspect. 15 (2002) 187–192.

[2] N.R. Farnsworth, R.O. Akerele, A.S. Bingel, D.D. Soejarto, Z. Guo. Medicinal plants in therapy, Bull. World Health Organ. 63 (1985) 965–981.

[3] G.M. Cragg, D.J. Newman. Natural products: A continuing source of novel drug leads, Biochim. Biophys. Acta. 1830 (2013) 3670-3695.

[4] A. Crozier, M.N. Clifford, H. Ashihara. Plants Secondary Metabolites, Ed. By Blackwell Publishing Ltd (2006).

[5] C. Rice-Evans, N. Miller, G. Paganga. Antioxidant properties of Phenolic compounds, Trends in Plant Science, 2 (1997) 152-159.

[6] A.M. Nuutilia, R. Puupponen-Pimia, M.Aarni, K.M. Oksman-Caldentey. Comparison of antioxidant activities of onion and garlic extracts by inhibition of lipid peroxidation and radical scavenging activity, Food Chem. 81 (2003) 485-493.

[7] J. Gershenzon, N. Dudareva. The function of terpene natural products in the natural world, Nature Chemical Biology, 3 (2007) 408-414.

[8] M. Wink. *In* "A Short History of Alkaloids. Alkaloids: Biochemistry, Ecology, and Medicinal Applications". M.F. Roberts and M. Wink (eds), Plenum Press, New York (1998) pp. 11-44.

[9] A. Meerow, D. Snijman. *In* "The family and Genera of Vascular Plants III. Flowering Plants Monocotyledons". K. Kubitzki (eds.), Springer-Verlag, Berlin (1998), pp. 83-110.

[10] A. Meerow, J. Francisco-Ortega, D. Kuhn, R. Schnell. Phylogenetic relationships and biogeography within the Eurasian clade of Amaryllidaceae based on plastid ndhF and nrDNA ITS sequences: lineage sorting in a reticulate area?, Syst. Bot. 31 (2006) 42-60.

[11] O. Hochino. *In* "The Alkaloids Chemistry and Biology". G.A. Cordell (eds.), Academic Press, San Diego (1998), pp. 323.

[12] Z. Jin. Amaryllidaceae and Sceletium alkaloids, Nat. Prod. Rep. 20 (2002) 606-614.

[13] A. Evidente, A. Andolfi, A.H. Abou-Donia, S.M. Touema, H.M. Hammoda, E. Shawky, A Motta. (-)-Amarbellisine, a lycorine-type alkaloid from *Amaryllis belladonna* L. growing in Egypt, Phytochemistry 65 (2004) 2113-2118.

[14] J. Bastida., R. Lavilla., F. Viladomat. Chemical and Biological aspects of *Narcissus* Alkaloids. *In* "The Alkaloids Chemistry and Biology". G.A. Cordell (eds.). Elsevier Academic Press: New York (2006) 87-179.

[15] C. W. Fennel, J. Van Staden. *Crinum* species in traditional and modern medicine, J. Etnopharmacol. 78 (2001) 15-26.

[16] Z. Jin. Amaryllidaceae and Sceletium alkaloids, Nat. Prod. Rep. 26 (2009) 363-381.

[17] J. Bastida., R. Lavilla., F. Viladomat. Chemical and Biological aspects of *Narcissus* Alkaloids. *In* "The Alkaloids Chemistry and Biology". G.A. Cordell (eds.). Elsevier Academic Press: New York (2006) 87-179

[18] E. Furusawa, H. Irie, D. Combs, W.C. Wildman. Therapeutic Activity of Pretazettine on Rauscher Leukemia: Comparison with the Related Amaryllidaceae Alkaloids, Chemoterapy 26 (1980) 36-45.

[19] I. Zupko. Antitumor Activity of Alkaloids Derived from Amaryllidaceae Species, In Vivo 23 (2009) 41-48.

[20] G. Ceriotti. Narciclasine: an Antimitotic Substance from *Narcissus* Bulbs, Nature 213 (1967) 595-596.

[21] D. Gonzalez, T. Martinot, T. Hudlicky. A Short Chemoenzymatic Synthesis of (+) Narciclasine, Tetrahedron Lett. 40 (1999) 3077-3080.

[22] D.H. Hua, S. Saha, D.J. Takemoto. Anticancer activities of 2,5,8,9-substituted 6-oxo 1,2,3,4,5,6-hexahydrophenanthridines on multi-drug-resistant phenotype cells, Anticancer Res. 17 (1997) 2435-2441.

[23] J. Corey-Bloom. Galanthamine: a review of its use in Alzheimer's disease and vascular dementia, International journal of clinical practice 57 (2003) 219-223.

[24] D.J. Mabberley. *In* "The Plant-book: A Portable Dictionary of the Vascular Plants*"* (2nd edn.). Cambridge University Press: Cambridge (1990).

[25] N.T.N. Tram et al. *Crinum* L. (Amaryllidaceae). Fitoterapia. 73 (2002) 183-208.

[26] W. Herbert. "Amaryllidaceae: preceded by an attempt to arrange the monocotyledonous orders, and followed by a treatise on cross-bred vegetables, and supplement". J. Ridgeway and sons: London (1837).

[27] J.C Cedron., M. Del Arco-Aguilar, A. Estevez-Braun, A.G. Ravelo. Chemistry and biology of Pancratium alkaloids. *In* "The alkaloids chemical and biology". G.A. Cordell (eds.). Academic Press: San Diego (2010) 1–37.

[28] F. Valsecchi. Le piante endemiche della Sardegna. Boll Soc Sarda Sci Nat 21 (1982) 427–41.

[29] L. Casu, F. Cottiglia, M. Leonti, A. De Logu, E. Agus, Y.C. Tse-Dinh et al. Ungeremine effectively targets mammalian as well as bacterial type I and type II topoisomerases. Bioorgan Med Chem Lett. 21 (2011) 7041–7044.

[30] E.P. Bareka, G. Kamari, D. Phitos. Cytogeographic study of the genus *Leucojum* (Amaryllidaceae) in Greece. Bocconea 16 (2003) 529-536.

[31] A. Pavlov. Galanthamine production by *Leucojum aestivum in vitro* systems. Process Biochemistry 42 (2007) 734–739.

[32] M. Savona, L. Minuto, B. Ruffoni, P .Profumo, S. Carli. *Leucojum nicaeense* Ard.: application of the in vitro culture to preserve the biodiversity. http://agris.fao.org/ ID=IT2005601967 (2004).

[33] B. Halliwell. Free radicals and antioxidants: updating a personal view. Nutr. Rev. 70 (2012) 257-265.

[34] A.A. Alfadda, R.M. Sallam. Reactive Oxygen Species in Health and Disease. J Biomed Biotechnol. 2012 (2012) 1-14.

[35] L.Landi, D. Fiorentini, M.C. Galli, J. Segura-Aguilar, R.E Beyer. DT-Diaphorase maintains the reduced state of ubiquinones in lipid vesicles thereby promoting their antioxidant function. Free Radic Biol Med. 22 (1997) 329-335.

[36] A.A. Podtelezenhnikov et al. Molecular insights into the pathogenesis of Alzheimer's disease and its relationship to normal aging. Plos One. 6 (12) (2011) e29610.

[37] M. Goedert, M.G. Spillantini. A century of Alzheimer's disease. Science 314 (2006) 777–781.

[38] S.C. Waring, R.N. Rosenberg. Genome-wide association studies in Alzheimer disease. Arch Neurol. 65 (2008) 329–334.

[39] F.A. Schmitt, D.G. Davis, D.R. Wekstein, C.D. Smith, J.W. Ashford, et al. "Preclinical" AD revisited: neuropathology of cognitively normal older adults. Neurology. 55 (2000) 370–376.

[40] W. Stöcker, F. Grams, U. Baumann, P. Reinemer, F.X. Gomis-Rüth. The metzincins topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases. Protein Sci. 4 (1995) 823–840.

[41] M.D. Sternlicht, G. Bergers. Matrix metalloproteinases as emerging targets in anticancer therapy: status and prospects. Emerging Ther. Targets.4 (2000) 609–633.

[42] M.D. Sternlicht, L.M. Coussens,. T.H. Vu, Z. Werb. Biology and regulation of the matrix metalloproteinases. In: Clendeninn, N.J..; Appelt, K., editors. Matrix Metalloproteinase Inhibitors in Cancer Therapy. Totowa, N.J.: Humana; (2001) 1-37.

[43] C.M. Overall. Matrix metalloproteinase substrate binding domains, modules and exosites. In: Clark, IM., editor. Matrix Metalloproteinase Protocols. Totowa, NJ: Humana; (2001) 79-120.

[44] D.R. Edwards. The tissue inhibitors of metalloproteinases (TIMPs). In: Clendeninn, NJ.; Appelt, K., editors. Matrix Metalloproteinase Inhibitors in Cancer Therapy. Totowa, NJ: Humana; (2001) 67-84.

[45] N.P. Kadoglou, C.D. Liapis. Matrix metalloproteinases: contribution to pathogenesis, diagnosis, surveillance and treatment of abdominal aortic aneurysms. Curr Med Res Opin. 20 (2004) 419–432.

[46] A.C. Newby. Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. Physiol Revv. 85 (2005) 1–31.

[47] M.L. Lindsey. MMP induction and inhibition in myocardial infarction. Heart Failure Revs. 9 (2004) 7–19.

[48] L.A. Cunningham., M. Wetzel, G.A. Rosenberg. Multiple roles for MMPs and TIMPs in cerebral ischemia. Glia. 30 (2005) 329–339.

[49] P.S. Burrage, K.S. Mix, C.E. Brinckerhoff. Matrix metalloproteinases: role in arthritis. Front Biosci. 11 (2006) 529–543.

[50] R. Marshall, G. Bellingan, G. Laurent. The acute respiratory distress syndrome: fibrosis in the fast lane. Thorax. 53 (1998) 815–817.

[51] E. Barrantes, M. Guinea. Inhibition of collagenase and metalloproteinases by aloins ans aloe gel. Life sciences. 72 (2004) 843-850.

[52] L. Sartor, E. Pezzato, I. dell'Aica, R. Caniato, S. Biggin, S. Garbisa. Inhibition of matrix-proteases by polyphenols: chemical insights for anti-inflammatory and anti-invasion drug design. Biochemical Pharmacology. 64 (2002) 229-237.

[53] Y.J. Kim, H. Uyama. Tyrosinase inhibitors from natural and synthetic source: structure, inhibition mechanism and perspective for the future. Cell. Mol. Life Sci. 62 (2005) 1707-1723.

[54] T.S Chang. An Updated Review of Tyrosinase Inhibitors. Int. J. Mol. Sci. 10 (2009) 2440-2475.

[55] E. Frenk. Treatment of melisma with depigmenting agents. In : Melasma: New Approaches to Treatment. Martin Dunitz, London, (1995) 9-15.

[56] M. Friedman. Food browning and its prevention: an overview. J. Agric. Food. Chem. 44 (1996) 631-653.

[57] M. Sugumaran. Molecular mechanism for culticular sclerotization. Adv. Insec. Physiol. 21 (1988) 179-231.

[58] WHO, World Health Report 2003, World Health Organization, Geneva, Swizerland, WHO publications Office, 2003, 1-50.

[59] M. Iwu, A.R. Duncan, C.O. Okunji. In: Janick J. (ed.), New antimicrobials of Plant Origin. Perspective on new crops and new uses. Alexandria, VA, ASHS Press, (1999) 457-462.

[60] M.M Cowan. Plants products as antimicrobial agents. Clin. Microbiol. Rev. 12 (1999) 564-582.

[61] V.G. Jr Fowler, J.M. Miro, B. Hoen, et al. *Staphylococcus aureus* endocarditis: a consequence of medical progress. JAMA. 293 (2005) 3012–3021.

[62] M.A. Pfaller, G.J. Moet, S.A. Messer, R.N. Jones, M. Castanheira. *Candida* bloodstream infections: comparison of species distributions and antifungal resistance patterns in community-onset and nosocomial isolates in the SENTRY Antimicrobial Surveillance Program, 2008–2009. Antimicrob Agents Chemother. 55 (2011) 561- 566.

[63] H. Wisplinghoff, T. Bischoff, S.M. Tallent, H. Seifert, R.P. Wenzel, M.B. Edmond. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from aprospective nationwide surveillance study. Clin Infect Dis. 29 (2004) 309–317.

[64] K. Martin, E. Ernest. Herbal medicines for treatment of bacterial infections: a review of controlled clinical trials. J. Antimicrob. Chemoter. 51 (2003) 241-246.

[65] G.A Ravishankar,. S. Ramachandra Rao. Biotechnological production of phyto-pharmaceuticals. J. Biochem.Mol. Biol. Biophys. 4 (2000) 73-102.

[66] G. Hansen, M.S. Wright. Recent advances in the transformation of plants. Trends Plant Sci. 4 (1999) 226-231.

[67] J. Bastida, R. Lavilla, F. Viladomat. Chemical and biological aspects of *Narcissus* alkaloids, in: G.A Cordell (eds.), the alkaloids chemical and biology, Elsevier Scientific Publishing, Amsterdam. (2006) 87-179.

[68] J.C. Coll, B.F. Bowden, The application of vacuum liquid chromatography to the separation of terpene mixtures, J. Nat. Prod.49 (1986) 934-936.

[69] A. Venditti, M. Mandrone, A.M. Serrilli, A. Bianco, C. Iannello, F. Poli, F. Antognoni. Dihydroasparagusic Acid: Antioxidant and Tyrosinase Inhibitory Activities and Improved Synthesis. J. Agric. Food. Chem. 61 (2013) 6848−6855.

[70] H.E. van Wart ,D.R.A. Steinbrink. Continuous Spectrophotometric Assay for *Clostridium hystolyticum* Collagenase. Anal. Biochem. 113 (1981) 225-231.

[71] M. Mandrone et al. Collagenase Inhibition measured througt a modified method using collagen as a substrate. Analytical Biochem. *Sumbitted.*

[72] T. Murashige, F. Skoog. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15 (1962) 473–497.

[73] N. Unver, G. I. Kaya, C. Werner, R. Verpoorte, B. Gozler. Galanthindole: A new Indole Alkaloids from *Galanthus plicatus* ssp. *Byzantinus*. Planta Medica 69 (2003) 869-871.

[74] S. Berkov, A. Pavlov, V. Georgiev, J. Weber, T. Bley, F. Viladomat, J. Bastida, C. Codina. Changes in apolar metabolites during *in vitro* organogenesis of *Pancratium maritimum*. Plant Physiology and Biochemistry 48 (2010) 827-835.

[75] A. Latvala, M.A. Onur, T. Gozler, A. Linden, B. Kivcac, M. Hesse. Alkaloids of *Galanthus elwesii*. Phytochemistry 39 (1995) 1229–1240.

[76] S. Kobayashi, M. Kihara, K. Yuasa, Y. Imakura, T. Shingu, A. Kato. Alkaloids constituents of *Leucojum aestivum* L. (Amaryllidaceae). Chem. Pharm. Bull. 33 (1985) 5258–5263

[77] E. N. Frankel, S. Huang, J. Kanner, J. Bruce German. Interfacial phenomena in the evalutation of antioxidants: Bulk oils vs emulsions. J. Agric. Food Chem. 42 (1992) 1054-1059.

[78] S. López, J. Bastida, F. Viladomat, C. Codina Acetylcholinesterase inhibitors activity of some Amaryllidaceae alkaloids and *Narcissus* extracts. Life Sci. 71 (2002) 2521–2525.

[79] G.M Bores, F.P. Huger, W. Petko, A.E. Mutlib, F. Camacho, D.K. Rush. Pharmacological evaluation of novel Alzheimer's disease therapeutics: acetylcholinesterase inhibitors related to galanthamine. J Pharmacol Exp Ther. 277 (1996) 728–738

[80] J.P de Andrade., S. Berkov, F. Viladomat, C. Codina, J.A.S. Zuanazzi, J. Bastida. Alkaloids from *Hippeastrum papilio*. Molecule 16 (2011) 7097–7104.

[81] B. Madham, V. Subramaninan, J. Raghava Rao, B. Unni Nair, T. Ramasami. Stabilization of collagen using plant polyphenol: Role of catechin. International Journal of Biological Macromolecules 37 (2005) 47–53

[82] S. C. Kim et al. Hordenine, a single compound produced during barley germination, inhibits melanogenesis in human melanocytes. Food Chemistry. 141 (2013) 174-181.

[83] S.Ghosal, S.K Singh, Y. Kumar, S. Unnikrishnan, S. Chattopadhyay. The Role of Ungeremine in the Growth-Inhibiting and Cytotoxic Effects of Lycorine: Evidence and Speculation. Planta Medica 28 (1998) 114-116.

[84] J.Bastida, S. Berkov, L. Torras, N.B. Pigni, J.P. de Andrade, V. Martinez, C. Codina, F. Viladomat. Chemical and biological aspects of Amaryllidaceae alkaloids. In Recent Advances in Pharmaceutical Sciences, Munoz-Torrero D (ed.). Transworld Research Network: Kerala, India; (2011) 65-100

[85] S.A. Adesanya, T.A. Olugbade, O.O. Odebiyi, J.A. Aladesanmi. Antibacterial Alkaloids in *Crinum jagus*. Pharmaceutical Biology. 30 (1992) 303-307.

[86] V. Sharma, P. Kumar, D. Pathak. Biological importance of the indole nucleus in recent years: A comprehensive review. J. Heterocyclic Chem. 47 (2010) 491-502.

[87] L. Cheesman, J.J. Nair, J. van Staden. Antibacterial activity of crinane alkaloids from *Boophone disticha* (Amaryllidaceae). J. Ethnopharmacol. 140 (2012) 405-408.

[88] J. L. Rodriguez-Tudela et al. EUCAST Definitive Document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts. Clin Microbiol Infect 14 (2008) 398–405.