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Evaluation of bioactive potential of a secondary metabolite produced by *Penicillium nordicum*

Agata Wierzbińska

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Supervised by
Paula Rodrigues
Isabel C.F.R. Ferreira

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ABSTRACT

Fungi of the genus *Penicillium* are promising sources of bioactive substances. Fungal strains isolated from poorly characterized habitats are currently under active investigation as potential producers of biologically active compounds. *Penicillium nordicum* is mostly known by its ability to produce the mycotoxin ochratoxin A in several protein- and salt-rich food matrices like processed meat and cheese, but it can also produce an array of other secondary metabolites. In a previous study, strains of this species were found to produce, under specific growth conditions, one unidentified compound in apparently big amounts, without producing ochratoxin A.

The aim of the present study was to evaluate the bioactive properties of this compound in terms of antioxidant, antimicrobial, cytotoxic and phytotoxic properties. For that purpose, the fungus was grown on chorizo-based medium and the aqueous extract of the compound was used for further tests.

Pestalotin was the predominant compound in the fungal extract, with questiomycin and 7-hydroxypestalotin also being found at lower concentrations, as detected by Liquid Chromatography-Mass Spectrometry (HPLC-MS/MS) based multi-metabolite method.

The extract did not show any cytotoxic activity in non-tumor porcine liver primary culture cells and human tumor cell lines. The antibacterial effect on human clinical isolates and antifungal effect on foodborne and environmental isolates was also not significant. However, it showed significant effects on seed germination as well as on root and shoot growth of tomato, lettuce, wheat and oat. The root growth inhibition ranged from 65.9% to 100%, and the shoot growth was reduced in 22.7% to 92%, being tomato the most sensitive plant.

The extract can be considered as a potential bioherbicide by its phytotoxic effect, without noticeable negative effects on human and animal cells and also on the environment. Further tests on herbicidal potential are now under development.

Keywords: *Penicillium nordicum*, cytotoxicity, antioxidant, antimicrobial, bioherbicide

RESUMO

Espécies fúngicas do género *Penicillium* são fontes promissoras de substâncias bioativas. Estirpes isoladas de habitats pouco caracterizados são atualmente sujeitos a forte investigação como potenciais fontes destas substâncias.

Penicillium nordicum é uma espécie reconhecida pela sua capacidade para produzir a micotoxina ocratoxina A em matrizes alimentares ricas em proteína e sal. No entanto, é também produtora de uma alargada gama de outros metabolitos secundários. Num estudo prévio, foi identificada em estirpes desta espécie a capacidade de, sob determinadas condições de incubação, produzir um composto desconhecido em quantidades aparentemente elevadas, sem produzir ocratoxina A.

O objetivo do presente estudo foi avaliar as propriedades bioativas deste composto, nomeadamente propriedades antioxidantes, antimicrobianas, citotóxicas e fitotóxicas. Para o efeito, o fungo foi produzido em meio de cultura à base de chouriço e o extrato aquoso foi usado para os testes necessários.

Após análise multi-metabolito por Cromatografia Líquida – Espetrometria de Massa (HPLC-MS/MS), pestalotina foi o composto encontrado no extrato em maior quantidade, sendo os compostos questiomicina e 7-hidroxipestalotina encontrados em menores concentrações.

O extrato não mostrou atividade citotóxica em cultura células primárias de fígado de porco não-tumorais ou em linhas celulares humanas tumorais. O efeito antibacteriano em isolados clínicos humanos e antifúngico em isolados de origem alimentar e ambiental não foi significativo. No entanto, foi detetado efeito fitotóxico significativo ao nível da germinação de sementes e do crescimento de raízes e rebentos de tomate, alface, trigo e aveia. A inibição do crescimento radicular variou entre 65.9% e 100%, e o crescimento do rebento foi inibido em 22.7% a 92%. O tomate foi a espécie mais sensível ao extrato.

Pelo efeito fitotóxico observado neste estudo, o extrato pode ser considerado um potencial bioherbicida, sem efeitos negativos significativos para células animais e para o ambiente. O potencial bioherbicida do extrato continua em estudo.

Palavras-passe: *Penicillium nordicum,* citotoxicidade, antioxidante, antimicrobiano, bioherbicida

I. INTRODUCTION

I.1. Framework

Fungi of the genus *Penicillium* are promising sources of bioactive substances. Fungal strains isolated from poorly characterized or completely uncharacterized habitats are currently under active investigation as potential producers of biologically active compounds. These habitats include ecological niches in which microorganisms are continuously exposed to stressful influences (Kozlovskii et al., 2015).

Exometabolites produced by different types of microorganisms may find multiple applications in different fields of industry. Currently, the pharmaceutical industry is regarded as the major application field for products from fungi. Many of fungal metabolites have entered the clinical pipeline in view of being exploited as novel drugs (Nicoletti & Trincone, 2016).

Penicillium nordicum is a mycotoxinogenic fungus, usually contaminating protein rich food with high levels of NaCl, which is capable of producing big number of exometabolites, known also as secondary metabolites (Rodríguez et al., 2014). During testing secondary metabolites produced by *Penicillium nordicum* strains it was discovered that, under certain growth conditions, and concomitantly with the production of the well-known metabolite ochratoxin A (OTA), the fungus was also producing one unidentified compound in apparently big amounts. This compound was considered a good candidate for further tests for bioactivity in terms of potential industrial applications.

To avoid the simultaneous production of other exometabolites with the compound of interest, it is essential to adjust the environmental conditions of compound production. After a virtually pure extract of the compound is obtained further tests can then be performed. However, since *P. nordicum* is a well-known mycotoxin-producing fungus, toxicity tests need to be done for the extract. Cytotoxicity tests are very important to be performed for new compounds in terms of determining their possible influence on other cells, either human or animal.

In terms of food or pharmaceutical industry applications of new, unknown compounds, some tests are also interesting in order to determine their potential antioxidant, anti-inflammatory, antifungal and antimicrobial activity. Some compounds produced by microorganism can also find applications in agriculture as herbicides or insecticides.

I.2. Objectives

The general aim of this project was to identify a secondary metabolite produced by *P. nordicum* and characterize it in terms of bioactivity.

To achieve this purpose, several tests were performed:

1. Optimization of the production process;

2. Elucidation of the extract composition by LC-MS (Liquid Chromatography-Mass Spectrometry);

3. Evaluation of the bioactive properties of the extract: cytotoxicity (with tumor and non-tumor cells), antimicrobial (against bacteria and fungi), antioxidant (DPPH free radicals scavenging activity, reducing power, inhibition of β-carotene bleaching and lipid peroxidation inhibition) and phytotoxicity (effect on seed germination and plantlet growth).

II. LITERATURE REVIEW

II.1. *Penicillium nordicum* and production of secondary metabolites

Species from the genus *Penicillium* are the second biggest producers of secondary metabolites (first is *Aspergillus*), with around 3.8 metabolites per species. Secondary metabolites (exometabolites) are small molecules produced during morphological and chemical differentiation that are outward directed. Unlike primary metabolites (endometabolites), that are fluctuating in concentration (the fluxome) or are transformed into other endometabolites, secondary metabolites are usually located in or on the cell wall and accumulated. Endometabolites are typical for almost all species of fungi (and most other kinds of organisms) but exometabolites are specific of certain species. Their most important feature is that environmental factors like light, pH, redox potential, temperature, water activity and also exometabolites from other species play a regulatory effect on regulatory proteins for exometabolite expression in the fungus (Frisvad, 2015).

Secondary metabolites are often regarded as biochemical markers for the classification of some species of fungi, and can provide useful information for the ascription of isolates displaying controversial morphological features. Also, the biologically active metabolites of *Penicillium* may have useful properties that can be exploited for developing new pharmaceuticals (Nicoletti et al., 2007; Nicoletti and Trincone, 2016).

Penicillium nordicum is one of mycotoxinogenic species from the genus *Penicillium* which is usually contaminating protein-rich food with high levels of NaCl. It is usually found on dry-cured meat products like: cured ham, salami, but also on salted fish and, occasionally, on cheese and jam (Ferrara et al., 2016). *P. nordicum* is capable of producing big number of secondary metabolites.

The exometabolites produced by *P. nordicum* with their bioactivity already known are presented in Table II.1.

Table II.1. Exometabolites produced by *Penicillium nordicum* and their bioactivity (Frisvad et al., 2004; Rodríguez et al., 2014)

Extrolite biosynthetic family	**Activity**
Verrucolones	Herbicidal
Pestalotins	Herbicidal
Ochratoxin A (OTA)	Mycotoxin (Nephrotoxic, Hepatotoxic, Immunotoxic)
Sclerotigenin	Antiinsectan
Viridic acid	Mycotoxin

Some of these metabolites are toxic to animals in low amounts, and are known as mycotoxins. The best known mycotoxin produced by *P. nordicum* is ochratoxin A (OTA), which can be detected in both mycelium and spores, as well as in contaminated food products. OTA is a potent mycotoxin, with nephrotoxic, nephrocarcinogenic, teratogenic, neurotoxic and immunotoxic activities. It is also involved in porcine and chicken nephropathy and it is suspected to be an important etiological factor in human Balkan endemic nephropathy, as in the occurrence of tumors of urogenital tract (Ferrara et al., 2016). It has been classified into Group 2B (possible human carcinogen) by the International Agency for Research of Cancer (IARC, 1993) and the Commission of European Communities fixed maximum admissible levels in several foodstuffs (EC, 2006, 2010).

The production of OTA by *P. nordicum*, like other toxins for different species of fungi, is dependent on growth substrates and environmental conditions (Vipotnik et al., 2017).

II.2. Optimization of compounds production processes

By definition, the production of secondary metabolites in fungi is dependent on growth substrates and environmental conditions. Fungal species are specifically associated to certain habitats, plants, animals or other organisms. There they produce exometabolites in response to the challenges in the particular habitat. Light, pH, redox potential, temperature, water activity, carbon sources, nitrogen sources, iron starvation, and exometabolites from other species can all have an effect on the regulatory proteins for exometabolites expression in a fungus, usually by stimulating the expression of gene clusters that would otherwise be silent (Frisvad, 2015).

The manipulation of the environment and nutrition of the microorganisms had shown substantial impacts on the quantity and diversity of secondary metabolite production. The optimization of the microbial culture may have great effects on the results of natural products

screening program. For the full metabolomic diversity exploitation of one or few microorganisms, the methodical modification culture growing conditions (OSMAC: one strain; many metabolites) approach is commonly used. However for a large-scale screening program it becomes problematic because application a suite of culture conditions for even one factor (media, pH, temperature, etc.) to every screened organism becomes hard and impractical (VanderMolen et al., 2013).

In a study presented by Medina et al. (2015) it was proven that different salt concentrations in the culture medium influenced the growth of *P. nordicum* and *A. ochraceus* and their exometabolites production (in this case OTA). It was shown that in high salt concentrations, where water activity was relatively low, the growth of *P. nordicum* and OTA production were better than on media with low salt concentrations with high water activity. For other fungal species those growth conditions represent environmentally stressed conditions and give the fungi from genera *Penicillium* and *Aspergillus* an ecological advantage to colonize those types of environments.

Another study proving that environmental conditions influence secondary metabolites production by fungi was presented by Kozlovskii et al. (2016). In this study *Penicillium* strains grown under different conditions (acidic, neutral and alkaline) shown to produce metabolites of different structural types.

For industrial applications of newly discovered bioactive compounds it is essential to optimize the production of a target bioactive compound at an industrial scale. As the addition to laboratory tests of different incubation conditions for microorganisms the discipline of metabolic engineering has increasingly been applied to the secondary metabolite studies to boost commercial production of target molecules. The main objective of this is to overproduce chemicals that are valuable to mankind from microbial or mammalian cells. It attempts to systematically understand and engineer a cell's metabolic network at a systems level. Systems metabolic engineering has been already applied to platform production strains such as *Escherichia coli* and *Saccharomyces cerevisiae* for the production of various chemicals, biofuels and biopolymers (Kim et al., 2016).

II.3. Structural elucidation of chemical compounds

Introduction of spectrometric tests in the new natural products discovery allows structural information to be used to identify and filter out any known compounds that are present in an extract. This way of natural products discovery is called 'structure-based' method. Thanks to this method it is possible to elucidate the structure of the chemical compounds and submit this information to databases that relate structure with bioactivity. By using this method, some of the identified compounds will be associated with confirmed bioactivity but it is also possible to identify compounds for which no bioactivity can be found (Henke & Kelleher, 2016).

Methods frequently used are structure-based methods. First of all, extracts from microbial or plant sources are screened for bioactivity and only if an extract displays activity, it is fractionated and the resulting fractions are then rescreened for that same activity. Later the compound is purified and then nuclear magnetic resonance spectroscopy (NMR) or high-resolution mass spectrometry (HRMS) are performed (Henke & Kelleher, 2016).

Together witch bioactivity tests as the base for a primary screen, the spectrometric or spectroscopic data has recently emerged as a viable option to screen for new natural products and they are used to determine already known compounds. The most common methods of detection for early stages are: ultraviolet-visible spectroscopy (UV/Vis) that provides information on chromophores present in a compound; NMR that provides information on chemical environment and connectivity within a molecule through NMR-active isotopes (e.g., ^{1}H, ^{13}C, ^{15}N); mass spectrometry (MS) that provides the mass of compounds and, if the instrument is capable of fragmentation, the masses of subsequent fragment ions. Very often those techniques are coupled to obtain more information about the compound. The most efficient method is MS or tandem MS because often only nanograms of material are needed to perform the test (Henke & Kelleher, 2016).

Recently, LC-MS technique is frequently used in terms of natural extracts composition analysis and trace -level determination of food contaminants. Combination of MS with LC (liquid chromatography) is considered as the best technique for quantification and semi quantitative screening of natural products. Although, GC (gas chromatography) in combination with MS is still frequently used in analyses of volatile, non- polar small molecules, LC-MS allows to perform more sensitive and selective determination of other more polar or ionic molecules. The goal of the use of LC-MS in initial screening is to determine presence of large number of compounds in large number of samples and to provide

a rapid analysis without generating false-negative results with small frequency of false-positive ones. Screening techniques with use of LC-MS may be targeted or non-targeted. Targeted methods are used to determine a list of already known analytes by comparison to the list of selected compounds, it usually also use reference standards. This approach is limited to the list of selected compounds and do not always present all compounds of interest. For full screening of metabolites, non-targeted analysis is used and allows to detect unexpected and unknown compounds (Hird et al. 2014).

II.4. Industrial applications of newly identified compounds

Many metabolites and compounds produced by bacteria and fungi find their applications in different fields of industry, for example, food or pharmaceutical industries. However, before introduction of those compounds to any field of industry, some fundamental bioactivity tests must be run in terms of determining the toxicity of the compound and also the potential influence on plant, animal and human health. Those tests include: antifungal, antimicrobial, antioxidant, anti-inflammatory, antitumor and phytotoxic potential tests.

Species of *Penicillium* (eg. *Penicillium marinum* and *Penicillium dravuni*) and *Talaromyces* are regarded as a fruitful investigational ground for the finding of novel bioactive compounds, leading to the discovery of blockbuster drugs, such as the antibiotic penicillin and the anticholesterolemic agent compactin, miscellaneous antitumor products, and mycotoxins contaminating food (Nicoletti & Trincone, 2016).

II.4.1. Antifungal potential

Pathogenic fungi cause tremendous economic losses in agriculture worldwide, in all pre-harvest, harvest and post-harvest stages of crops and fruits as well as in animal husbandry. Mycotoxins contaminate and spoil foods and feedstuffs and can be deleterious for domestic animals and, through the food chain, even for humans. The extensive use of chemical fungicides in plant and animal protections can raise the resistance of fungi to those chemicals and can also contaminate agricultural food products.

Biological control of fungal contamination based on microbial antagonists can be investigated as a possible way to improve food safety without affecting quality and properties of typical dry-cured meat products. Sometimes to prevent the food products from

exometabolites of one organism, the other organism or its metabolite is added. Due to the application of other harmless and non-toxic organism or metabolite, the growth of the pathogenic organisms or the production of toxic compounds can be inhibited (Virgili et al., 2012).

In a study, presented by Virgili and collaborators, it was shown that some genera of non-pathogenic yeasts have antagonistic effect on fungi from genera *Penicillium* that grow on dry-cured meat products. The isolated yeasts were able of growing to high populations in conditions of temperature, pH, moisture, water activity and salt typical of dry-cured ham surface. Selected yeasts were added to dry-cured meat products as starter cultures to enrich the volatile compound profile. The antagonistic potential of yeasts against *Penicillia* has been already considered before for different food matrixes, such as cereals, coffee, fruits or fermented food like wine, cheese and yogurt (Virgili et al., 2012).

Because of this there is an urgent need for the development of new-type and effective antimycotic agents. Antifungal proteins produced as exometabolites by non-cytogenic fungi can be promising and exploitable candidate biofungicides. Antifungal proteins produced by filamentous fungi are small, basic (lysine- and/or arginine-rich), cysteine-rich peptides stabilized by three to four disulfide bridges. Those proteins bind to the negatively charged plasma membrane of the sensitive organisms and may cause membrane permeabilization, elevation of intracellular ROS levels and, finally, the initiation of programmed cell death. The best studied antifungal proteins produced by filamentous fungi are AFP, secreted by *Aspergillus giganteus*, PAF from *Penicillium chrysogenum* and Anafp from *Aspergillus niger* (Leiter et al., 2017).

Also the production of antimycotic substances is one of the biochemical mechanisms regulating antagonism between fungi. For soil fungi, for instance, it may influence fungistasis and the properties of certain soils toward plant pathogens. Fungi-toxic extrolites are considered to have their inhibitory effect in the soil environment not only on occurrence of a direct hyphal contact, but also over a small distance, either by diffusion or by volatilization. Many *Penicillium* species have developed competitive abilities based on the release of antifungals that may affect the proliferation of other pathogenic fungi. It was documented that *P. canescens* and *P. janczewskii* show antagonistic activity toward *Rhizoctonia solani*. Griseofulvin that was first extracted from a strain of *Penicillium griseofulvum* and is considered as an antimycotic drug (Nicoletti & Trincone, 2016), was produced by both

P. canescens and *P. janczewskii* and inhibited the hyphal growth of *R. solani*. (Nicoletti et al., 2007).

II.4.2. Antibacterial potential

Fungi can be a source of natural antibiotics, which can be low-molecular weight (LMW) and high-molecular weight (HMW) compounds. LMW compounds are mainly secondary metabolites such as sesquiterpenes and other terpenes, steroids, anthraquinone and benzoic acid derivatives, and quinolines, but also primary metabolites such as oxalic acid. HMW compounds mainly include peptides and proteins (Alves et al., 2012).

Antibiotics act in several ways, by interfering in metabolic processes or in the organism structures. The mechanism of action is mostly related with interferences in the synthesis of the cell wall, modification of plasmatic membrane permeability, interferences in chromosome replication, or in protein synthesis (Alves et al., 2012).

Despite the huge diversity of antibacterial compounds, bacterial resistance to first-choice antibiotics has been drastically increasing. Diseases that were easily healed are now becoming a serious problem. Because of that, natural resources have been exploited in the last years to find new natural sources of alternative antimicrobials. Fungi proved to be a promising alternative (Alves et al., 2012).

Mycophenolic acid is famous as the first known fungal antibiotic, discovered as a product of a strain of *Penicillium brevicompactum*. It has antibiotic, antiviral, and cytostatic properties and is applied in medicine as an immunosuppressive drug. Gliotoxin and chaetoglobosins, first characterized from respectively *Gliocladium fibriatum* and *Chaetomium globosum* and later in few *Penicillium* species, show strong antibiotic/cytotoxic properties with a series of interesting effects on human tumor cells (Nicoletti & Trincone, 2016).

II.4.3. Antioxidant potential

Reactive oxygen species (ROS), like free radicals and other oxidants [e.g., O_2•–, $^1O_2$, HO•, NO•, $ONOO^-$, HOCl, RO(O)•, LO(O)•], cause major problems for human health (Prior et al., 2005).

ROS are produced in metabolic and physiological processes due to oxidative chain-reactions, but they can be neutralized via enzymatic and non-enzymatic anti-oxidative

mechanisms. Under some conditions, the increase in oxidants and decrease in antioxidants cannot be prevented, and the oxidative/anti-oxidative balance shifts toward the oxidative status (Ozcan, 2004).

The production of ROS is increased in several diseases such as: diabetes, obesity, hypertension, atherosclerosis or cancer. Researchers are focused on finding natural sources of substances that could strengthen the natural antioxidant response of human organism and protect humans from the harmful effects of oxidant compounds. Substances with antioxidant properties are mostly found in plants but can be also produced by microorganisms (Cybul & Nowak, 2008).

In biological systems, there are four general sources of antioxidants: enzymes (superoxide dismutase, glutathione peroxidase, and catalase); large molecules (albumin, ceruloplasmin, ferritin, other proteins); small molecules (ascorbic acid, glutathione, uric acid, tocopherol, carotenoids, (poly)phenols); and some hormones (estrogen, angiotensin, melatonin, etc.). Individual antioxidants may sometimes act by multiple mechanisms in a single system or by a different single mechanism depending on the reaction system. Because of multiple reaction characteristics and mechanisms as well as different phase localizations are usually involved, no single attempt will accurately reflect all of the radical sources or all antioxidants in a mixed or complex system. That is why there is no simple universal method by which assessing antioxidant capacity (AOC) can be measured accurately and quantitatively (Prior et al., 2005). The natural antioxidants are summarized in Figure II.1.

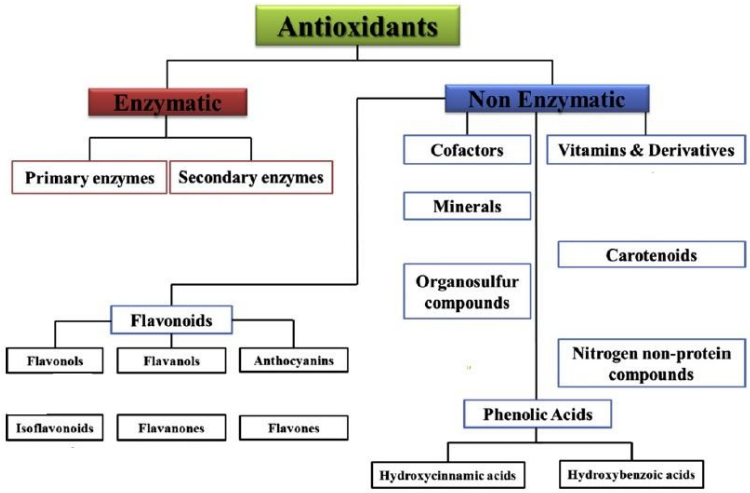

Figure II.1. Natural antioxidants separated in classes (Carocho & Ferreira, 2013).

II.4.4. Anti-inflammatory potential

Inflammation is a physiological response to injury, characterized by loss of function and pain, heat, redness and swelling. It is usually associated with the pathogenesis of diseases such as diabetes, arthritis, obesity, metabolic syndrome, cancer and several cardiovascular diseases. Uncontrolled production of inflammatory mediators has been known to cause several cell damages and also initiate the inflammation process. Natural products are good resources for development of therapeutic compounds with anti-inflammatory potential and without or low toxic effects (Oludemi et al., 2016).

Macrofungi (fruiting bodies, mycelia or their submerged fermentation broth) as well as microfungi are rich in several bioactive compounds, either if wild, edible or cultivated species. These bioactive metabolites include phenolic compounds, terpenoids, polysaccharides, lectins, steroids, glycoproteins and some lipid components. In several studies it has been shown that mushrooms extracts and/or their secondary metabolites have antioxidant, antitumour, antimicrobial, immunomodulator, antiatherogenic, hypoglycemic and anti-inflammatory activities (Oludemi et al., 2016).

It was discovered that some species of fungi like *Aspergillus niger* and *Cunninghamella elegans* are able to metabolize isoflavones and 4'-fluoroisoflavone. Isoflavones are the compounds that can be commonly found in numerous plants, especially from *Leguminosae* but recent studies revealed that some microorganisms can also produce flavones and isoflavones through the *de novo* biosynthesis (Lee et al., 2014).

Isoflavones are molecules with many biological activities, including antioxidant, anti-inflammatory, antitumor, and antifungal activities. Some isoflavones have also protective roles against pathogenic fungi and bacteria. Despite the anti-microbial roles, several fungi and bacteria can metabolize these compounds. The use of biological systems in transformation processes is a good method to increase the diversities of natural products in drug discovery, drug applications, food industry or enzyme preparation (Lee et al., 2014).

II.4.5. Antitumor potential

Carcinogenesis is a process in which progressive genetic changes occur leading to malignant transformation. Due to the technology development, currently it is possible to explore molecular pathways, cancer-associated genes and tissue architecture. This knowledge provided the basis for most cancer-preventive intervention strategies and particularly for one

of the strategies, chemoprevention – the use of drugs, biologicals and nutrients to prevent the development of cancer (i.e. to inhibit, delay or reverse carcinogenesis) (Ferreira et al., 2010).

As mentioned before, fungal fruiting bodies, fungal mycelium or the culture fluid in which the mycelium has been cultivated may all be explored for biological activity. Some species of edible higher Basidiomycetes have shown the inhibition effect on the growth of different tumor cell lines. There are approximately two hundred species of higher Basidiomycetes that were found to have this activity. Moreover, both cellular components and secondary metabolites of a large number of mushrooms have been shown to affect the immune system. Those properties can be used in treatment of various diseases, including cancer, immunodeficiency diseases (including AIDS) or generalized immunosuppression after drug treatment (Ferreira et al., 2010).

Macrofungi are sources of powerful new pharmaceutical products, including compounds with potential antitumor and immunostimulating properties, such as LMW and HMW compounds (Ferreira et al., 2010).

Not only macrofungi are showing interesting antitumor properties. As mentioned gliotoxin and chaetoglobosins detected in a few *Penicillium* species show a series of interesting effects on human tumor cells. Also 3-O-methylfunicone, secondary metabolite produced by strains of *Penicillium pinophilum* (=*Talaromyces pinophilus*), was characterized for its cytostatic properties on a number of human tumor cell lines. It showed effects on cytoskeletal organization, cell cycle progression, the expression of pro-apoptotic genes, the inhibition of markers of tumor progression, and other mechanisms suppressing cell proliferation/migration (Nicoletti & Trincone, 2016).

II.4.6. Phytotoxic potential

Weeds infest economically important crops causing losses in agrarian production, forests, and ornamental heritages. Weed pests, including parasitic plants are one of the most serious agricultural and environmental problems due to competition with the growth of agrarian crops and forest plants by competing for water, nutrients, light and by the serious obstacles they represent for agronomic activities. There are many weed management strategies in agriculture production, including mechanical, cultural, chemical, and biological strategies. The use of chemical pesticides may have effects on plant toxicity, herbicidal resistance and environmental changes. Because of this new strategies based on the use of

natural products are being developed. The most popular strategy is the use of fungal phytotoxins alone as natural herbicides and/or in combination with fungal producers in a more efficient, integrated management (Cimmino et al., 2015).

Fungal phytotoxins are secondary metabolites that have the ability to induce disease symptoms in agrarian and forest plants and in weeds. They belong to different classes of naturally occurring compounds: aromatics, aminoacids, coumarins and isocoumarins, cytochalasans, ethanones, furopyrans, nonenolides, oxazatricycloalkenones, pyrones, spirophytotoxins, terpenes, trichothecenes, and some others with a complex and original carbon skeleton (Cimmino et al., 2015).

Phytopathogenic fungi are the promising sources of toxins that may play a role in plant diseases and preservation of microbial species in their natural habitat. Herbicidal-resistant weeds are nowadays a big problem in weed control because of the big number of weed biotypes resistant to herbicides that constantly increases by the continuous use of the same products for years. New herbicides with different mode of action are needed and phytotoxins produced by fungi are the promising ones (Souza et al. 2017).

III. MATERIALS AND METHODS

III.1. Compound production

P. nordicum was grown on seven media and under two temperatures to determine the best conditions for the production of the compound of interest, i.e., the production of high amounts of compound with reduced production of other metabolites, mostly ochratoxin A. Test conditions were selected based on previous studies (Vipotnik et al., 2017).

The base medium was produced by boiling 5% of traditional chorizo (T) in water and filtering the meat extract through cheese cloth. Then, seven different media were prepared by adding different amounts of NaCl and glycerol, as described in Table III.1.

A suspension of *P. nordicum* spores was prepared by mixing a loop full of spores from a 7-day old culture in 500 µL of water with 0.05% of Tween 80. All plates were inoculated by three-point inoculation with 3 µL of the spore suspension and incubated for 15 days at 15 ºC and 20 ºC (Table III.1).

Table III.1. Conditions of *Penicillium nordicum* incubation in terms of culture medium (percentage of NaCl and glycerol added to the chorizo base medium) and temperature.

Condition	NaCl	Glycerol	Temperature of incubation (°C)
TP 1.1	3%	-	20
TP 1.2			15
TP 2.1	-	2%	20
TP 2.2			15
TP 3.1	3%	2%	20
TP 3.6			15
TP 4.1	-	-	20
TP 4.2			15
TP 5.1	6%	-	20
TP 5.2			15
TP 6.1	-	8%	20
TP 6.2			15
TP 7.1	6%	8%	20
TP 7.2			15

After the incubation period, 3 plugs of agar from each plate (corresponding to 0.5 g of medium) were cut with the aid of a cork borer and mixed with 1.5 mL of methanol for 60 minutes, with vortexing every 15 minutes, for the extraction of the compound. The obtained

extracts were analyzed using an HPLC system (Smartline, Knauer, Berlin, Germany) coupled to a fluorescence detector (FP-2020, Jasco, Easton, MD, USA) set to λex 330 nm and λem 463 nm and using the Clarity 2.4 Software (DataApex, Prague, Czech Republic). The compounds were separated using an isocratic elution with a reverse-phase C18 column (100 mm × 4.6 mm, Merck Chromolith Performance, Darmstadt, Germany) at 35 °C (7971 R Grace oven). The mobile phase consisted of a mixture of acetonitrile/water/acetic acid (70:29.5:0.5, *v*/*v*/*v*), with a flow rate of 0.8 mL/min. The injection volume was 10 µL.

The amount of the detected (unknown) metabolites was calculated as relative concentration equivalent of OTA, in ng/mL, by using an already existing calibration curve of OTA standard as reference. For the purpose of the study, the condition producing the biggest amount of compound with the smallest amount of OTA (TP 4.1; base medium incubated at 20 ºC) was selected to produce the compound in the necessary amounts for the following tests. For this, big amounts of extract were obtained as previously described. The extract was evaporated with the use of a rotative evaporator at ~60 ^{o}C and stored at 4 ºC until further use. The same medium but without fungal inoculation was used as negative control.

III.2. Extract characterization

The extract was analyzed at the University of Natural Resources and Life Sciences in Department for Agrobiotechnology in Austria by performing HPLC-ESI-MS/MS based multi-mycotoxin method using an Agilent 1290 HPLC coupled to an Applied Biosystems QTrap Mass spectrometer. Screening and Quantification was performed in the Selected Reaction Monitoring (SRM) mode. The LC-MS/MS protocol has been published in Malachova et al. (2014), and has been extended to cover 710 metabolites overall. Two MRM (Multiple Reaction Monitoring) transitions were acquired per analyte, which yields 4 identification points for unambiguous identification according to Commission Decision 2002/657/EC. In addition, the retention time and the intensity ratio of the two MRM transitions has to agree with the related values of an authentic standard within 2.5 % rel. and. 30% rel., respectively. The sample was diluted 1+19 using acetonitrile/water 1/1 + 1% acetic acid and 5 µL were directly injected.

External calibration was performed using serial dilutions of a multi-analyte stock solution. Limits of detection (LOD) in the standards were calculated from the signal-to-noise

ratios (LOD = 3 * S/N). From these values, the LODs in the diluted liquid sample were calculated taking into account the dilution factor.

III.3. Evaluation of bioactive properties of extract

III.3.1 Evaluation of antioxidant activity

III.3.1.1. DPPH radical scavenging activity

The extract was dissolved in methanol at 5 mg/mL, and further diluted into sequential concentrations. DPPH (2,2-diphenyl-1-picryl-hydrazyl) radical-scavenging activity was evaluated using an ELX800 microplate reader. In each well it was added 30 µL of each concentration of the extract and 270 µL of methanolic solution containing DPPH radicals (6×10^{-5} mol/L). The reduction of DPPH was determined after 1 h of reaction in the dark, by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation:

$$\%RSA=[(A_{DPPH} - A_S)/ A_{DPPH}] \times 100$$

where A_{DPPH} is the absorbance of DPPH solution and A_S is the absorbance extract solution (Carocho et al. 2014).

III.3.1.2. Reducing power

The reducing power (RP) assay was also performed using the Microplate Reader mentioned above. Each concentration of the extract (0.5 mL) was mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% (w/v), 0.5 mL). The mixtures were incubated at 50 ^{o}C for 20 min and trichloroacetic acid (10% (w/v), 0.5 mL) was added. The mixture (0.8mL) was transferred into the wells, as also deionized water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL). The reducing power was evaluated by measuring the capacity of the extract to reduce Fe^{3+} to Fe^{2+} by measuring the absorbance at 690 nm (Barros et al. 2010).

III.3.1.3. Inhibition of β-carotene bleaching

Inhibition of β-carotene bleaching (βC) was evaluated through the β-carotene/linoleate assay. In this assay, the bioactive samples neutralize the linoleate free radicals, reducing β-carotene discoloration (Carocho et al. 2014a).

A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two milliliters of prepared solution was transferred into round-bottom flask and evaporated at 40 ^{o}C. After evaporation under vacuum, Tween 80 emulsifier (400 mg), lionelic acid (40 mg) and distilled water (100 mL) were added with vigorous shaking. Different concentrations of extract (0.2 mL) were put in the test tubes and prepared emulsion was added (4.8 mL). Control was prepared by adding the extraction solvent (0.2 mL) instead of the extract into the emulsion (4.8 mL). As soon as the emulsion was added, initial absorbance was measured at 470 nm and tubes were incubated in a water bath at 50 ^{o}C with agitation (100 rpm) for 2 h. Reduction of discoloration was measured after 2 h at 470 nm and calculated using the formula:

$$\% = [A_{\beta C\_2h} / A_{\beta C}] \times 100$$

where $A_{\beta C\_2h}$ is the absorbance after 2 h of assay and $A_{\beta C}$ is the initial absorbance (Barros et al. 2010).

III.3.1.4. Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)

The thiobarbituric acid reactive substances (TBARS) assay used porcine brain homogenates that react with ascorbic acid to create the malondialdehyde - thiobarbituric acid (MDA-TBA) (Carocho et al. 2014a).

The porcine brain was homogenized with Tric-HCl buffer (20 mM, pH 7.4), to produce 1:2 (w/v) brain tissue homogenate which was later centrifuged at 3000 g for 10 min. Different concentrations of extract (0.2 mL) were transferred into test tubes and $FeSO_4^+$ (10 μM, 0.1mL), ascorbic acid (0.1 mM, 0.1 mL) and supernatant of brain tissue homogenate (0.1 mL) were added. Two controls were performed, one with extraction solvent (methanol) and other with the Tris-HCl buffer (20 mM, pH 7.4) with addition of reagents mentioned before (except extract). All tubes were incubated at 37 ^{o}C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% (w/v), 0.5 mL), followed by thiobarbituric acid (TBA, 2% (w/v), 0.38 mL). Then the mixture was heated at 80 ^{o}C for 20 min and centrifuged at 3000 g for 10 min.

The inhibition of lipid peroxidation was measured by colorimetric assay at 532 nm using the formula:

$$\% = [(A - B)/A] \times 100$$

where A and B are respectively the absorbance of the control and of the extract solution (Barros et al. 2010).

The results of the antioxidant activity were expressed in EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay).

III.3.2. Evaluation of the cytotoxic properties in tumor and non-tumor cells

The cytotoxicity was tested on four human tumor cell lines: MCF7 (breast adenocarcinoma), NCI H460 (lung carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). The density of cells on 96-well plates was 7.5×10^3 cells/well for MCF7 and HCT15, and 1.0×10^4 cells/well for HeLa and HepG2. Further, different concentrations of extract (8, 2, 0.5 0.125 mg/mL) diluted in distilled water were added to prepared cells. The cytotoxicity was also tested on non-tumor cells, using a cell culture prepared from fresh liver of porcine (PLP2), which was slaughtered in a certified facility. A phase-contrast microscope was used to monitor the growth of the cell cultures. They were sub-cultured and plated in 96 well plates (density of 1.0×10^4 cells/well). DMEM (Dulbecco's Modified Eagle Medium) medium was used, with 10% of FBS (Fetal Bovine Serum), penicillin (100 U/mL) and streptomycin (100 µg/mL). Ellipticine was used as a standard, and the results were expressed in GI_{50} values in µg/mL (sample concentration that inhibited 50% of the net cell growth) (Carocho et al., 2014b).

III.3.3. Evaluation of the antibacterial activity

To determine the antibacterial activity, Gram-negative (G-) and Gram-positive (G+) bacteria were used. The G- bacteria used were: *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Morganella morganii*, and G+ bacteria were: *Enterococcus faecalis, Listeria monocytogenes* and S*taphylococcus aureus*. A fresh overnight culture of bacteria was adjusted with a spectrophotometer at 625 nm to a concentration of 1×10^5 CFU/mL (colony forming units). The extract solutions (in concentrations of: 20, 10, 5, 2.5, 1.250, 0.625 and 0.315 mg/mL) were pipetted into the wells containing Tryptic Soy Broth (TSB) (100 µL). After that, the inoculum (10 µL) was added to all the wells. The microplates were incubated for 24 h at 37 °C, followed by the addition of iodonitrotetrazolium chloride (INT) (0.2 mg/mL, 40 µL) and by incubation at 37 °C for 1 h. MIC (minimal inhibitory concentration) was determined visually by a colorimetric microbial viability assay based on

the reduction of INT color and compared with the positive control for each bacterial strain. Streptomycin and ampicillin were used as standards, while medium extract was used as a negative control (Carocho et al. 2015).

III.3.4. Evaluation of the antifungal activity

The antifungal activity test was performed using the following microfungi: *Aspergillus fumigatus*, *Aspergillus ochraceus*, *Aspergillus versicolor*, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus flavus*, *Penicillium funiculosum*, *Penicillium ochrochloron*, *Penicillium verrucosum* var. *cyclopium* and *Trichoderma viride*.

The micromycetes were maintained on malt agar (MA) and the cultures were stored at 4°C and sub-cultured once a month. The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 µL per well. The inocula were stored at 4 °C for further use. Dilutions of the inocula were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculum. The minimum inhibitory concentrations (MIC) were determined by a serial dilution of the extract using 96-well microplates. The extract solutions were added to the broth malt medium with the fungal inoculum. The microplates were incubated for 72 h at 28 °C. The lowest concentrations without visible growth (using a binocular microscope) were defined as the MIC. The minimum fungicidal concentrations (MFC) were determined by serial sub-cultivation of 2 µL of the previous suspensions in microtiter plates containing 100 µL of malt broth per well and further by incubation for 72 h at 28 °C. The lowest concentration with no visible growth was defined as the MFC, indicating 99.5% killing of the original inoculum. Culture medium was used as a negative control, while bifonazole and ketoconazole were used as standards (Carocho et al. 2015).

III.3.5. Preliminary evaluation of phytotoxicity

Herbicidal activity test was performed on Schulz Medium (SM; Table III.2) with the addition of the extract in the concentration of 15 mg/mL dissolved in distilled water. SM was used as negative control. Plant seeds used for the test were: *Solanum lycopersicum* (tomato), *Lactuca sativa* (lettuce), *Triticum aestivum* (common wheat) and *Avena sativa* (oat). All seeds

used were previously surface-disinfected in 10% bleach for 2 min and washed twice with sterile water.

Table III.2. Content of Schulz Medium (SM).

Reagent	Concentration
$KH_2PO_4 \cdot 3H_2O$	0.25 g/L
KCl	0.25 g/L
$MgSO_4 \cdot 7H_2O$	0.25 g/L
$Ca(NO_3)_2$	1.00 g/L
EDTA	20.0 mg/L
agar	8.00 g/L

Two millilitres of each media were poured in large test tubes and two seeds of each plant were inoculated on the media. Each plant was tested in four repetitions on each medium. Tubes were incubated in an *in vitro* culture room with photoperiod with variable temperature (17 h of light at 25 ^{o}C and 7 h of dark at 15 ^{o}C) for 10 days. Seed germination and growth of each plant were monitored daily. Root and shoot growth were visually measured from the outside of the tube after 2, 3, 4, 5, 8, 9, 10 and 14 days of incubation. At the end of this period, plants were taken out of the tubes, separated from the medium by slight heating and measured with the aid of a ruler. The number of roots and leaves was also registered. Results were expressed in % of growth reduction of roots and shoot for each plant.

Statistical analysis was performed using IBM® SPSS® Statistics v.22.0 software (Armonk, NY: IBM Corp.). The variables under study did not show a normal distribution, therefore the non-parametric statistical U-Mann Whitney test was used for comparison of means. In all cases, statistical significance was established at $p \leq 0.05$.

IV. RESULTS AND DISCUSSION

IV.1. Compound production

Fungal growth was observed at all tested media. The growth of *P. nordicum* on chorizo-based medium after an incubation period of 14 days at 20 ºC is shown in Figure IV.1.

Figure IV.1. Growth of *Penicillium nordicum* on chorizo-based medium (5% chorizo extract), without NaCl and glycerol added (TP4.1), after 14 days of incubation at 20 ºC.

HPLC analysis showed that *P. nordicum* grown on different media produced different types of metabolites and at different amounts (Figure IV.2). The retention time of the compound of interest was around 4.45 min. Under some conditions, OTA was also detected, at retention time 2.45 min, as compared with the standard solution. The relative amounts of all detected metabolites (as OTA equivalents, in ng/mL), including that of the compound of interest and OTA, are presented in Figure IV.3.

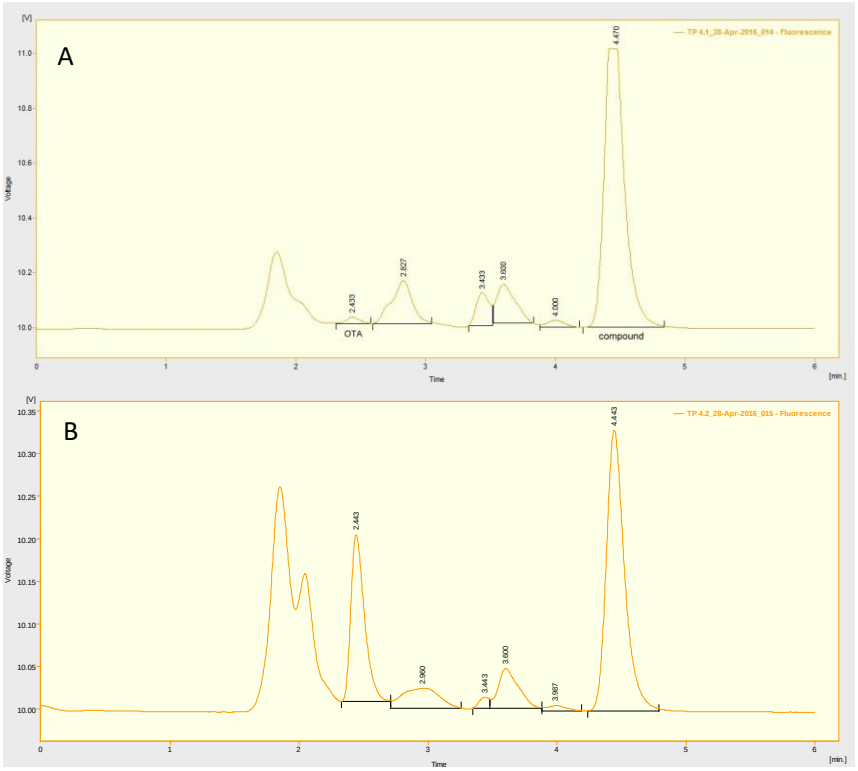

Figure IV.2. Comparison of two HPLC- FLD chromatograms where OTA and compound of interest were produced in different amounts under different conditions: (A) TP 4.1 - medium containing 5% chorizo extract, incubated in 20 ^{o}C; (B) TP 4.2 - medium containing 5% chorizo extract, incubated at 15 ^{o}C.

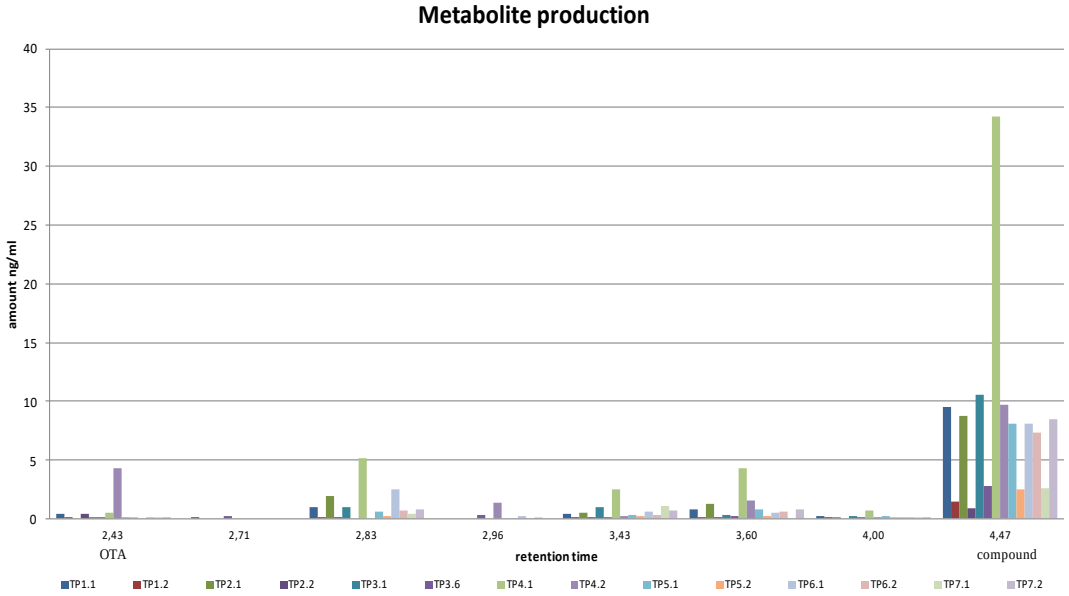

Figure IV.3. Relative concentration of metabolites (as OTA equivalents, in ng/mL) produced by *Penicillium nordicum* on the different tested conditions.

The highest amount of the target compound was produced on TP4.1 medium, containing 5% of chorizo extract (no NaCl and glycerol added) and incubated at 20 ^{o}C, with only negligible amounts of OTA being produced (Figure IV.2A). Under some other culture conditions (TP 2.2, TP 4.2; Figure IV.2B), OTA production was detected. The condition TP4.1 was considered as the most adequate to be used for the production of the compound in big amounts for further tests, because it was the condition where the highest amount of compound and lowest amount of OTA were produced, with the advantage of being the most cost-effective one (no NaCl and glycerol added; less energy spent on refrigeration).

IV.2. Extract characterization

The analysis of the extract by the multi-metabolite detection method detected three metabolites present at concentrations higher than the quantification limit of the method: Pestalotin (52 ng/mL), Questiomycin (39.4 ng/mL) and 7-Hydroxypestalotin (15.3 ng/mL). All other investigated metabolites were below their limit of detection (Appendix I). Figure IV.4 represents the LC-MS chromatogram of the extract, showing the three detected metabolites (Figure IV.4A).

The enhanced mass spectrum of the protonated species ($[M+H]^+$; *m/z*=215) of pestalotin and the corresponding product ion scan for the precursor ion (*m/z*=153) is shown in Figure IV.4B. Figure IV.5 shows the chemical structure of pestalotin.

Pestalotin (also known as compound LL-P880α; Kirihata et al., 1986), which was present in the extract in the biggest amount, was already known to be produced by *P. nordicum* and the compound is considered to have herbicidal activity (Frisvad, 2004) and to be a potent gibberellin-synergist on rice seedlings (Kirihata et al., 1996). Gibberellins are plant hormones that regulate growth and influence stem elongation and seed germination. In a study by Kimura et al. (1971) it was proven that the promotive effect of gibberellins on release of reducing sugar was enhanced by the simultaneous addition of pestalotin, promoting shoot growth of *Oryza sativa* (rice).

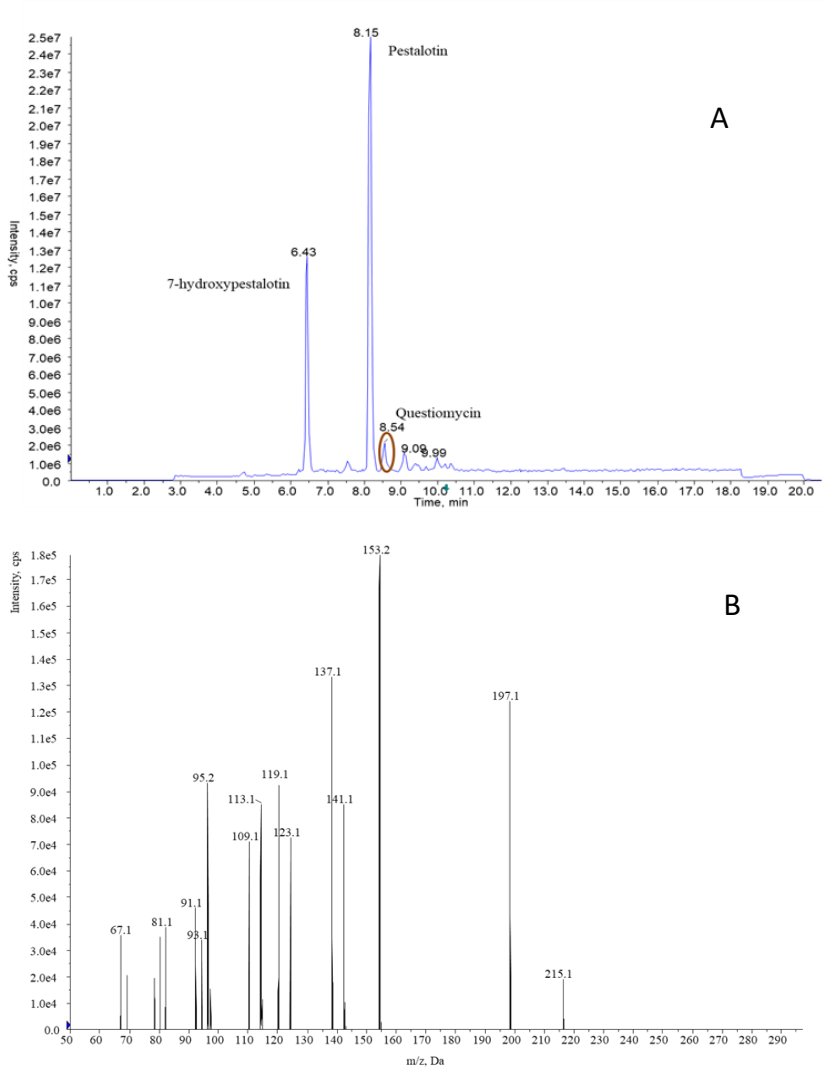

Figure IV.4. Enhanced product ion scan for the precursor ion *m/z*=215 (protonated species of pestalotin). (A) LC-MS chromatogram of the extract; (B) Enhanced Product Ion Spectrum of pestalotin (*m/z*=153).

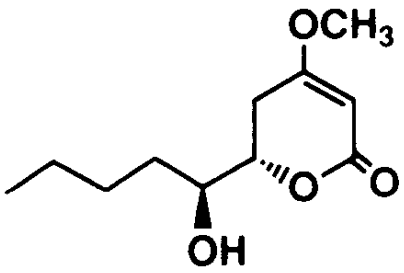

Figure IV.5. Chemical structure of pestalotin (LL-P880α) (Kirihata et al., 1996).

The enhanced mass spectrum of the protonated species ($[M+H]^+$; *m/z*=213) of questiomycin and the corresponding product ion scan for the precursor ion (*m/z*=156) is shown in Figure IV.6. Figure IV.7 shows the chemical structure of the compound.

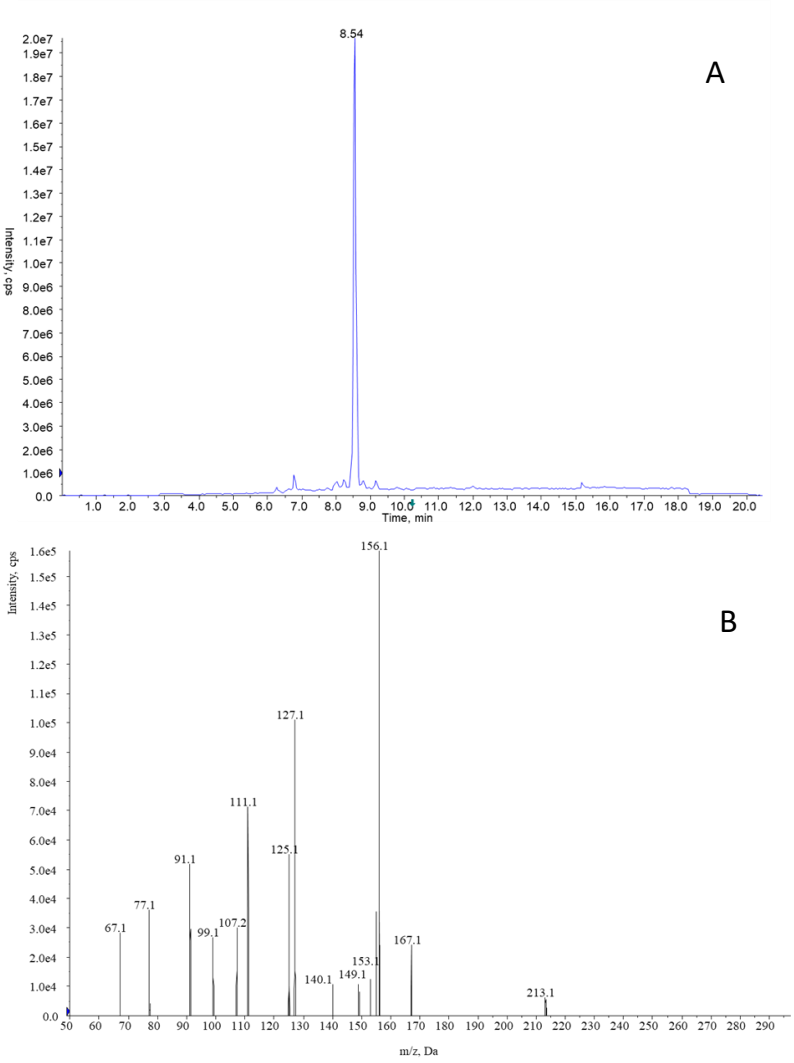

Figure IV.6 Enhanced Product Ion Scan for *m/z*=213 (protonated species of questiomycin). (A) LC-MS chromatogram of the enhanced product; (B) Enhanced Product Ion Spectrum of questiomycin (*m/z*=156).

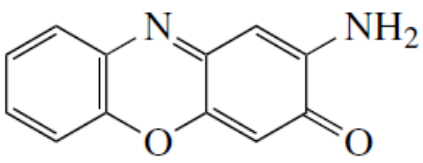

Figure IV.7. Chemical structure of Questiomycin A (2-amino-3H-phenoxazine-3-one) (Kozlovsky et al., 2004).

Questiomycin, the second most abundant compound detected in the extract, is a phenoxazine that was discovered in several strains of actinomycetes, bacteria, and the fungus *P. chrysogenum* Thom. Phenoxazinones of various structures are chromophore moieties of antibiotics produced by various actinomycetes. (Kozlovsky et al., 2004). Questiomycin shows weak activity against bacteria, fungi, plants and tumor cell lines, and inhibits aromatase and sulfatases. Questiomycin, like other phenoxazines, stimulates cell growth and turnover *in vitro*, an activity possibly related to their ability to form stable free radicals (www.scbt.com).

7-hydroxypestalotin ((-) - *(6S* ,I'*S, 2'R)* - *6* - *(1'*, 2'- Dihydroxypentyl) - 4- methoxy - 5,6-dihydropyrane-2-one, also known as LL-P880β), a minor analog metabolite of pestalotin, was the less abundant of the three quantifiable compounds detected in the extract. Its chemical structure is represented in Figure IV.8. The MS spectrum of 7-hydroxypestalotin was not obtained.

7-hydroxypestalotin was first isolated from an unidentified *Penicillium* species and in 1986 was isolated from a culture filtrate of *Penicillium citreo-viride* by Kimura and co-workers (Kimura et al. 1986). Unlike pestalotin, this compound hardly shows gibberellin synergist activity (Kirihata et al., 1996).

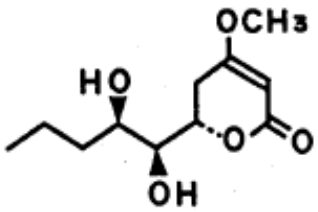

Figure IV.8. Chemical structure of 7-hydroxypestalotin ((-) - (6S ,I'S, 2'R) - 6 - (1', 2'- Dihydroxypentyl) - 4- methoxy - 5,6-dihydropyrane-2-one, LL-P880β) (Kirihata et al. 1996).

Technique used for determining extract composition was targeted LC-MS analysis. Extract was screened for unknown compounds and was compared to the list of reference compounds. However, because of the use of targeted approach, some compounds, since they were not present on the list of reference, might not be identified.

Non-targeted LC-MS analysis is more time consuming and requires more effort than targeted one but it allows to determine compounds in the extract that normally, with the use of

conventional targeted analysis, would not be determined. The disadvantage of non-targeted analysis is the quite big frequency of occurrence of false negatives because it is impossible to determine if a compound is sufficiently recovered during the analytical procedure or is not ionized as expected. For non- targeted analysis on unknown analytes the MS/MS analysis must to be performed separately in terms of selection of precursor ion from TIC (total ion current). Further chosen precursor ion must be fragmentized via CID (collision-induced dissociation) and the product ions are scanned. Targeted LC-MS approach is faster and allows rapid monitoring of hundreds of analytes. Targeted technique coupled with MS/MS is used to limit false positive results by searching against mass spectra libraries (Hird et al. 2014).

IV.3. Evaluation of bioactive properties of the extract

IV.3.1. Evaluation of antioxidant activity

The antioxidant activity of the extract was determined through various assays: DPPH scavenging activity, reducing power (RP) through Prussian-blue assay, inhibition of β-carotene bleaching, and inhibition of TBARS formation in brain cell homogenates.

The extract showed antioxidant activity only in RP assay: 0.5 of absorbance at 690 nm at a concentration of 2.5 mg/mL. For the other performed assay, it was not possible to determine the EC_{50} values (results presented in Table IV.1). In DPPH scavenging assay as well as in TBARS analysis, already on the microplates, no significant change of color, in comparison to positive controls, was noticed (Figures IV.9 - 10).

Table IV.1. Antioxidant activity of the extract

Antioxidant activity	**EC_{50} values [mg/mL]**
DPPH scavenging activity	> 5
Reducing power	2.5
β-Carotene bleaching inhibition	> 5
TBARS inhibition	> 5

Figure IV.9. DPPH scavenging analysis with the use of ELX800 microplate Reader (Bio-Tek Instruments, Inc.; Winooski, VT, USA); extract: wells 2-4 A-G, culture medium (negative control): wells 6-8 A-G; methanol (positive control): wells 2-4, 5-8 H.

Figure IV.10. TBARS assay analysis with the use of ELX800 microplate Reader (Bio-Tek Instruments, Inc.; Winooski, VT, USA); extract: wells 1-2 A-F, culture medium (negative control): wells 3-4 A-F; Tris (positive control): wells 5-6 A, methanol (positive control): wells 5-6 B.

In the study presented by Canturk et al. (2017) it was proven that among 82 isolates from *Penicillium* genus only 28.2% of them showed antioxidant activity in DPPH scavenging activity test. *P. flavigenum* was one of the species that showed the highest antioxidant activity effect.

Many secondary metabolites, with antioxidant activity, produced by microfungi from *Penicillium* sp. found their applications in different fields of industry in order to drug production (Canturk et al. 2017). For that reason compound extract obtained in this study from *P. nordicum* was examined in terms of antioxidant activity. Nonetheless obtained results proved that tested extract did not show antioxidant activity.

IV.3.2. Evaluation of the cytotoxic properties

Cytotoxicity was tested on four human tumor cell lines: MCF7 (breast adenocarcinoma), NCl H460 (lung carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). The results are presented in Table IV.2. The extract did not show activity against any of the tumor cell lines tested, up to the maximal tested concentration. Also no hepatotoxicity was detected for the PLP2 (non-tumor porcine liver cells).

Table IV.2. Cytotoxic activity of the extract performed on tumor and non-tumor cell lines.

Antitumor activity	**GI_{50} [µg/mL]**
MCF7	>400
NCl H460	>400
HeLa	>400
Hep G2	>400
Hepatotoxicity	
PLP2	>400

In a study by Luo et al. (2012), pestalotin and 7-hydroxypestalotin were detected in a fermentation broth of the endophytic plant fungus *Pestalotiopsis karstenii* isolated from stems of *Camellia sasanqua*. These compounds displayed strong activities against the cell line U-251 (human glioma cell line), with IC50 values of 2.5 and 12.0 µg/mL, respectively, but showed no activity against HeLa, A549, HepG2 and MCF-7 cell lines.

Many *Penicillium* species are well known for mycotoxins and antibiotic production that present cytotoxic effects and may have direct or indirect implications in cell division, showing the fundamental properties of typical antitumor products. However, some compounds such as citrinin, ochratoxin A, patulin, penicillic acid, alternariol and PR-toxin, produced by *Penicillium* sp. cannot be applied in pharmaceutical industry because of their high toxicity (Nicoletti et al. 2008).

In studies presented by Nicoletti et al. (2008, 2016), it was proven that many secondary metabolites produced by *Penicillium* sp. show significant antitumor activities. *P. nordicum* was not mentioned as one of the producers of antitumor extrolites. For that reason after obtaining extract of unknown secondary metabolite from *P. nordicum* it was

needed to check its potential cytotoxicity in terms of pharmaceutical applications. Nonetheless extract did not show any cytotoxic activities.

IV.3.3. Evaluation of antimicrobial activity against bacteria and fungi

The extract was tested against various Gram positive and Gram negative bacteria, and fungi. Medium extract was used as the negative control. MIC and MFC (for fungi) were determined and presented in Tables IV.3 and IV.4.

Table IV.3. Antibacterial activity of compound extract and culture medium; MIC- minimal inhibitory concentration; ESBL- spectrum extended producer of β- lactamases.

	MIC (mg/mL)	
Bacteria strains	**Test extract**	**Negative control (medium extract)**
Gram +		
Enterococcus faecalis	>20	>20
Listeria monocytogenes	>20	>20
Methicillin susceptible *Staphylococcus aureus*	>20	>20
Methicillin resistant *Staphylococcus aureus*	>20	>20
Gram -		
Escherichia coli ESBL	>20	>20
Escherichia coli	>20	>20
Klebsiella pneumoniae ESBL	>20	>20
Klebsiella pneumoniae	>20	>20
Morganella morganii	>20	>20
Pseudomonas aeruginosa	>20	>20

For antibacterial activity test extract did not show activity for the concentrations tested. However, in antifungal activity test, among all tested fungi, extract showed inhibitory and fungicidal activity against *A. ochraceus* and *T. viride*, with MIC value of 0.075 mg/mL and MFC value of 0.15 mg/mL for both fungi. For *A. versicolor, A. niger, A. terreus, A. flavus* and *P. ochrochloron*, the extract showed inhibitory and fungicidal activity higher than those observed by ketoconazole, but lower than bifonazole. For that reason, the extract was not considered to have significant antifungal activity against those strains.

Table IV.4. Antifungal activity of compound extract; MIC (mg/mL) - minimal inhibitory concentration; MFC - minimal fungicidal concentration; *MFC (mg/mL) were not determined due to insufficient amount of sample

Fungal strains	**Test Extract** MIC/MFC	**Neg. control (Medium extract)** MIC/MFC	**Bifonazole** MIC/MFC	**Ketoconazole** MIC/MFC
Aspergillus fumigatus	0.30/0.60	0.60/0.90	0.15/0.20	0.20/0.50
Aspergillus versicolor	0.15/0.30	0.30/0.90	0.10/0.20	0.20/0.50
Aspergillus ochraceus	0.075/0.15	0.20/0.60	0.15/0.20	1.50/2.00
Aspergillus niger	0.15/0.30	0.60/0.90	0.15/0.20	0.20/0.50
Aspergillus terreus	0.15/0.30	0.60/0.90	0.15/0.20	0.50/1.00
Aspergillus flavus	0.30/0.60	0.60/1.20	0.20/0.25	1.00/1.50
Trichoderma viride	0.075/0.15	0.30/0.60	0.15/0.20	1.00/1.00
Penicillium funiculosum	0.60/*	0.90/*	0.20/0.25	0.20/0.50
Penicillium ochrochloron	0.60/*	0.90/*	0.20/0.25	2.50/3.50
Penicillium verrucosum	0.90/*	1.20/*	0.10/0.20	0.20/0.30

Some fungi from genera *Penicillium* are able to produce very high number of antibiotic and antifungal compounds (Frisvad et al. 2004). In study presented by Frisvad et al. 2004 it was shown how big number of biologically active exometabolites is produced by *Penicillium* species.

In structure elucidation study it was determined that one of compounds present in quite big amounts was questiomycin, being known as an antibiotic. However antibacterial study did not show any antibiotic activity of the extract.

IV.3.4. Preliminary evaluation of phytotoxic activity

Phytotoxic activity of the extract was analyzed on a preliminary test on four different plants: two dicotyledonous - *Solanum lycopersicum* (tomato) and *Lactuca sativa* (lettuce) -, and two monocotyledonous - *Triticum aestivum* (common wheat) and *Avena sativa* (oat). Plantlets of the four plant species tested, obtained after 10 days of growth on SM (negative control, medium without extract) and SM-X (medium with extract added) are presented in Figure IV.11. The extract effect on the root and shoot growth is shown in Figures IV.12 and IV.13.

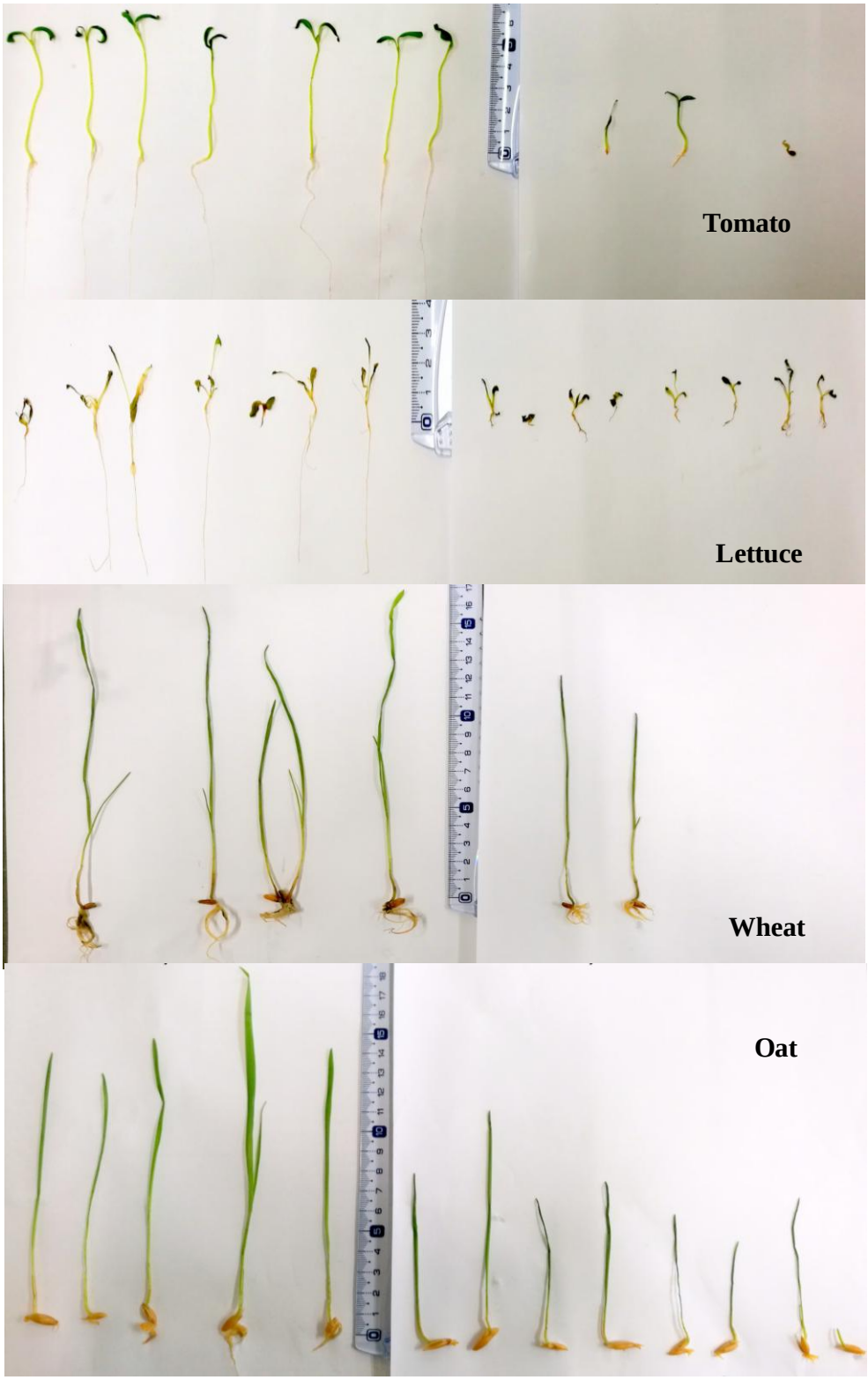

Figure IV.11. Plantlets of the four plant species tested, obtained after 10 days of growth on SM (left of the ruler) and SM with the extract (right).

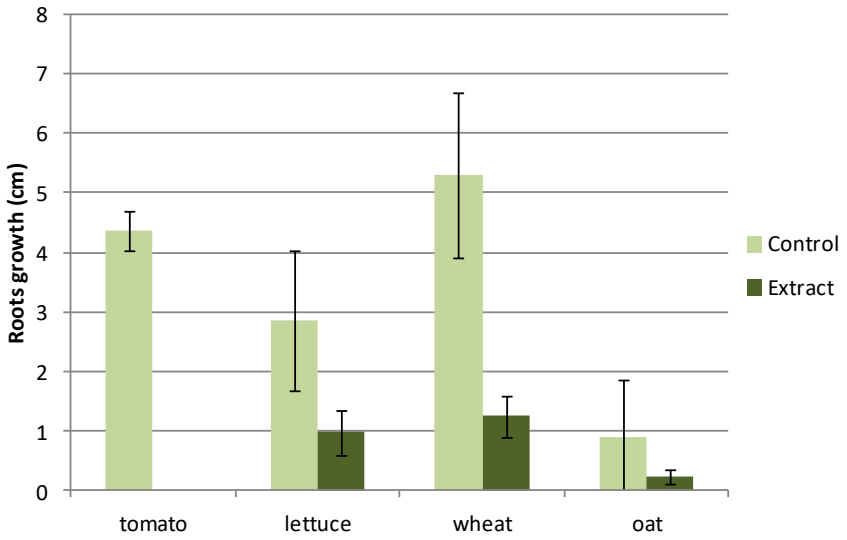

Figure IV.12. Effect of the extract on the roots growth of tested plants in comparison with control (SM).

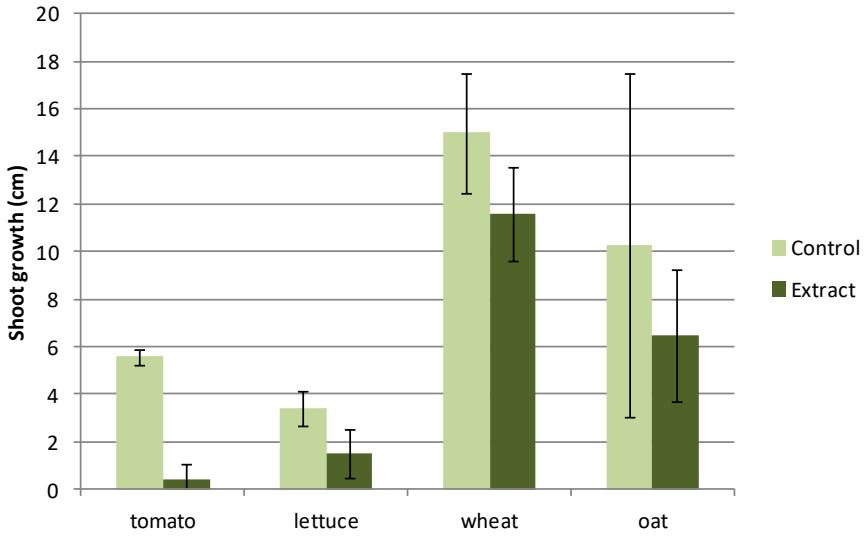

Figure IV.13. Effect of the extract on the shoot growth of tested plants in comparison with control (SM).

The root growth inhibition ranged from 65.9% to 100%, and the shoot growth was reduced in 22.7% to 92%, being tomato the most sensitive plant (Table IV.5). The standard deviation was calculated and it showed quite big values. That big deviation might be caused by some contaminations of the seeds and also lack of homogeneity between the seeds of each species.

Table IV.5. Comparison of root and shoot growth inhibition of tested plants in comparison to control (SM); n=6 (for each plant) ± sd

	root growth reduction [%]	shoot growth reduction [%]
tomato	100.0 ± 0.0	92.1 ± 11.0
lettuce	65.9 ± 12.8	55.9 ± 30.4
wheat	76.4 ± 6.7	22.7 ± 13.2
oat	75.3 ± 14.1	37.1 ± 27.1

Statistical analysis was performed to compare means in terms of influence of the extract on growth of roots and shoot of tested plants (Table IV.6). Statistical analysis was also performed to compare the growth reduction between the species (Table IV.7). Statistical significance was established at $p \leq 0.05$.

Table IV.6. Statistical analysis of means in terms of influence of the extract on roots and shoot growth of tomato, lettuce, wheat and oat.

	p-value			
	tomato	lettuce	wheat	oat
number of roots	0.001	1.000	0.279	1.000
number of leaves	0.001	0.040	0.161	0.345
length of roots	0.001	0.009	0.083	0.043
length of shoot	0.001	0.002	0.105	0.043

Table IV.7. Statistical comparison between species in terms of growth reduction.

	p- value	
	reduction of root length	reduction of shoot length
tomato x lettuce	0.001	0.007
tomato x wheat	0.005	0.032
tomato x oat	0.002	0.002
lettuce x wheat	0.289	0.293
lettuce x oat	0.113	0.269
wheat x oat	0.596	0.358

Statistical analysis showed that the extract has the significant influence on root and shoot growth of tomato, lettuce and oat, by inhibiting their growth. It also significantly influenced the number of roots and leaves of tomato and number of leaves of tomato and lettuce. Wheat appeared to be the most resistant to the inhibitory effect of the extract.

The comparison between species confirmed that the tomato was the most sensitive one, on the effect of the extract, between tested plants. The most significant difference between species in terms of growth inhibition of roots and shoot was between tomato and oat (p= 0,002).

Observed inhibitory effect was probably caused because of big amount of pestalotin in the extract (see IV.2.), that is considered as the potential herbicide (Frisvad, 2004) with gibberellin-synergist action (Kirihata et al., 1996). Basing on the results presented on Figures IV.10 and IV.11 it was determined that compound extract affected mostly roots of all tested plants.

Nowadays production of new bioherbicides is essential because of limited traditional chemical control options due to ecodegradation, health hazards, and the development of herbicide resistance in weeds (Souza et al. 2017). In the study presented by Souza et al. (2017) it was proven that 28 from 39 tested fungi showed herbicidal activity resulting in growth inhibition of aerial parts, reduction of height and fresh weight.

Fungal phytotoxins have the ability to induce disease symptoms in agrarian and forest plants and in weeds. They belong to different classes of naturally occurring compounds and are used as biocontrol agents to manage weeds on agriculture crops causing large necrosis on leaves and stems and also a retarded growth or death of plants (Cimmino et al. 2015). In the study of Cimmino et al. (2015) it was proven that most of tested fungal phytotoxins showed strong herbicidal activity without showing toxicity against terrestrial microorganisms and animals. It was also determined that the use of mixture of few phytotoxins except one showed better effects on weed growth inhibition also without showing antifungal, antibiotic and zootoxic activities. In comparison to the chemical herbicides, bioherbicides are safer for the environment simultaneously showing high herbicidal effects.

V. CONCLUSIONS AND PERSPECTIVES

Examined extract showed herbicidal activity without showing strong antimicrobial and cytotoxic properties. For that reason extract is considered to be a new promising bioherbicide. Further herbicidal activity assays are being performed on tested extract. It was shown that dicots (tomato and lettuce) are more vulnerable to inhibitory effects of the extract then monocots (wheat and oat). Since extract is considered as a potential herbicide, further toxicity analysis must to be performed against insects. It is very important in terms of possible agricultural applications.

Although many fungal metabolites showed potential herbicidal activity, their tests in greenhouses and in the open field are still very limited. Also the transfer to industry for large scale production and their application into practice as commercial products are still problematic due to lack of funding and low yields in production.

For the industrial scale-up of phytotoxins an optimization of the production process in the fermenter is needed. However, fermenters, widely used for the bacteria for the large scale antibiotics production, also need to be developed to avoid the adhesion of the mycelia masses to the walls and the poles of the bioreactor.

VI. REFERENCES

Alves M.J., Ferreira I.C.F.R., Dias J., Teixeira V., Martins A., Pintado M. (2012), A Review on Antimicrobial Activity of Mushroom (Basidiomycetes) Extracts and Isolated Compounds, *Planta Med*, 78, 1707–1718

Barros L., Oliveira S., Carvalho A.M., Ferreira I.C.F.R. (2010), In vitro antioxidant properties and characterization in nutrients andphytochemicals of six medicinal plants from the Portuguese folk medicine, *Industrial Crops and Products*, 32, 572–579

Canturk Z., Kocabıyık E., Ozturk N., İlhan S. (2017), Evaluation of Antioxidant and Antiproliferative Metabolites of *Penicillium flavigenum* Isolated from Hypersaline Environment: Tuz (Salt) Lake by Xcelligence Technology, *Microbiology*, 86, 346–354

Carocho M., Ferreira I.C.F.R. (2013), A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives, *Food and Chemical Toxicology*, 51, 15–25

Carocho M., Barros L., Bento A., Santos-Buelga C., Morales P., Ferreira I.C.F.R. (2014a), *Castanea sativa* Mill. Flowers amongst the Most Powerful Antioxidant Matrices: A Phytochemical Approach in Decoctions and Infusions, *BioMed Research International*, 7, Article ID 232956, 7 pages

Carocho M., Calhelha R.C., Queiroz M-J.R.P, Bento A., Morales P., Soković M., Ferreira I.C.F.R. (2014b), Infusions and decoctions of *Castanea sativa* flowers as effective antitumor and antimicrobial matrices, *Industrial Crops and Products*, 62, 42–46

Carocho M., Barros L., Calhelha R.C., Ćirić A., Soković M., Santos-Buelga C., Morales P., Ferreira I.C.F.R., (2015). *Melissa officinalis* L. decoctions as functional beverages: a bioactive approach and chemical characterization, *Food & Function*, 6, 2240–2248

Carocho M., Barros L., Barreira J.C.M, Calhelha R.C., Soković M., Fernández-Ruiz V., Santos Buelga C., Morales P., Ferreira I.C.F.R. (2016), Basil as functional and preserving ingredient in ‘‘Serra da Estrela” cheese, *Food Chemistry*, 207, 51–59

Cimmino A., Masi M., Evidente M., Superchi S., Evidente A. (2015), Fungal phytotoxins with potential herbicidal activity: chemical and biological characterization, *Natural Product Reports*, 32, 1629–1653

Cybul M., Nowak R. (2008), Przegląd metod stosowanych w analizie właściwości antyoksydacyjnych wyciągów roślinnych, *Herba Polonica*, 54, 68–78

E.C. (2006), Commission Regulation (EU) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs, *Official Journal of the European Union*, 20 December 2006.

E.C. (2010), Commission Regulation (EU) No 105/2010 of 5 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards ochratoxin A.

Ferrara M., Magistà D., Epifani F., Cervellieri S., Lippolis V., Gallo A., Perrone G., Susca A. (2016), Study of gene expression and OTA production by *Penicillium nordicum* during a small-scale seasoning process of salami., *International Journal of Food Microbiology*, 227, 51–55

Ferreira I.C.F.R., Vaz J.A., Vasconcelos M.H., Martins A. (2010), Compounds from Wild Mushrooms with Antitumor Potential, *Anti-Cancer Agents in Medicinal Chemistry*, 10, 424–436

Frisvad J.C. (2015) Taxonomy, chemodiversity, and chemoconsistency of *Aspergillus*, *Penicillium*, and *Talaromyces* species, *Frontiers in Microbiology*, 5, 773, 1–7

Frisvad J.C., Smedsgaard J., Larsen T.O., Samson R.A. (2004), Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*, *Studies in Mycology*, 49, 201–241

Henke M.T., Kelleher N.L. (2016), Modern mass spectrometry for synthetic biology and structure-based discovery of natural products, *Natural Product Reports*, 33, 942–950

Hird S.J, Lau B P.-Y., Schuhmacher R., Krska R. (2014), Liquid chromatography-mass spectrometry for the determination of chemical contaminants in food, *Trends in Analytical Chemistry*, 59, 59–72

IARC (1993), *Ochratoxin A (Group 2B).* In IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins, *International Agency for Research on Cancer*, 56, 489

Kim H.U., Charusanti P., Sang S.Y., Weber T. (2016), Metabolic engineering with systems biology tools to optimize production of prokaryotic secondary metabolites, *Natural Product Reports*, 33, 933–941

Kimura Y., Katagiri K., Inoue T., Tamura S. (1971), Isolation and Biological Activity of Pestalotin, a Gibberellin Synergist from *Pestalotia cryptomeriaecola*, *Agricultural and Biological Chemistry*, 35, 1313–1314

Kimura .Y, Hamasaki T., Nakajima H. (1986), Stereochemistry and Biological Activities of LL-P880γ, a Pestalotin Analogue, Produced by *Penicillium citreo-viride*, *Agricultural and Biological Chemistry*, 50, 1649–1650

Kirihata M., Ohe M., Ichimoto I., KimuraY. (1996), Synthesis and Biological Activity of LL-P880γ and Its Analogues, *Bioscience, Biotechnology, and Biochemistry*, 60, 677–679

Kozlovsky A. G., Zhelifonova V. P., Antipova T. V., Adanin V. M., Novikova N. D., Deshevaya E. A., Schlegel B., Dahse H. M., Gollmik F., Grafe U. (2004), *Penicillium expansum*, a Resident Fungal Strain of the Orbital Complex *Mir*, Producing Xanthocyllin X and Questiomycin A, *Applied Biochemistry and Microbiology,40, 291–295*

Kozlovskii A.G., Antipova T.V., Zhelifonova V.P. (2015), Biosynthesis of Biologically Active Low-Molecular Weight Compounds by Fungi of the Genus *Penicillium*, *Applied Biochemistry and Microbiology*, 51, 236–241

Lee J.-H., Oh E.-T., Chun S.-C., Keum Y.-S. (2014), Biotransformation of Isoflavones by *Aspergillus niger* and *Cunninghamella elegans*, *The Korean Society for Applied Biological Chemistry and Springer*, 57, 523−527

Leiter E., Gall T., Csernoch L., Pocsi I. (2017), Biofungicide utilizations of antifungal proteins of filamentous ascomycetes: current and foreseeable future developments, *BioControl*, 62, 125–138

Luo D.Q., Zhang L., Shi B.Z., Song X.M. (2012), Two New Oxysporone Derivatives from the Fermentation Broth of the Endophytic Plant Fungus *Pestalotiopsis karstenii* Isolated from Stems of *Camellia sasanqua*, *Molecules*, 17, 8554-8560

Malachová A., Sulyok M, Beltrán E., Berthiller F., Krska R. (2014), Optimization and validation of a quantitative liquidchromatography–tandem mass spectrometric method covering 295 bacterial and fungal metabolites including all regulated mycotoxins infour model food matrices, *Journal of Chromatography A*, 1362, 145–156

Medina A., Heydt S.M., Rodríguez A., Parra R., Geisen R., Magan N. (2015), Impacts of environmental stress on growth, secondary metabolite biosynthetic gene clusters and metabolite production of xerotolerant/xerophilic fungi, *Current Genetics*, 61, 325–334

Nicoletti R., Lopez-Gresa M.P., Manzo E., Carella A., Ciavatta M.L. (2007), Production and fungitoxic activity of Sch 642305, a secondary metabolite of *Penicillium canescens*, *Mycopathologia*, 163, 295–301

Nicoletti R., Ciavatta M.L., Buommino E., Tufano M.A. (2008) Antitumor extrolites produced by *Penicillium* species, *International Journal of Biomedical and Pharmaceutical Sciences*, 2, 1–23.

Nicoletti R., Trincone A. (2016), Bioactive Compounds Produced by Strains of *Penicillium* and *Talaromyces* of Marine Origin, *Marine Drugs*, 14, 37, 1–35

Oludemi T., Martins A., Barreiro M.F., Ferreira I.C.F.R. (2016), Anti-inflammatory potential of mushroom extracts and isolated metabolites, *Trends in Food Science & Technology*, 50, 193–210

Ozcan E. (2004), A novel automated method to measure total antioxidant response against potent free radical reactions, *Clinical Biochemistry*, 37, 112–119

Prior R.L., Wu X., Schaich K. (2005), Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements, *Journal of Agricultural and Food Chemistry*, 53, 4290–4302

Rodríguez A., Medina A., Córdoba J.J., Magan N. (2014), The influence of salt (NaCl) on ochratoxin A biosynthetic genes, growth and ochratoxin A production by three strains of *Penicillium nordicum* on a dry-cured ham-based medium, *International Journal of Food Microbiology*, 178, 113–119

Souza A.R.C., Baldoni D.B., Lima J., Porto V., Marcuz C., Machado C., Ferraz R.C., Kuhn R.C., Jacques R.J.S., Guedes J.V.C., Mazutti M.A. (2017), Selection, isolation, and identification of fungi for bioherbicide production, *Brazilan Journal of Microbiology*, 48, 101–108

VanderMolen K.M., Raja H.A., El-Elimat T., Oberlies N.H. (2013), Evaluation of culture media for the production of secondary metabolites in a natural products screening program, *AMB Express*, 3, 71, 7 pages

Vipotnik Z., Rodríguez A., Rodrigues P., (2017), *Aspergillus westerdijkiae* as a major ochratoxin A risk in dry-cured ham based-media, *International Journal of Food Microbiology*, 241, 244–251

Virgili R., Simoncini N., Toscani T., Leggieri M.C., Formenti S., Battilani P. (2012), Biocontrol of *Penicillium nordicum* Growth and Ochratoxin A Production by Native Yeasts of Dry Cured Ham, *Toxins*, 4, 68–82

Appendix I

Limits of detection of the investigates analytes (expressed as ng/mL sample taking into consideration the dilution factor; n.a.: not available due to lack of a quantitative reference standard)

Analyte	LOD (ng/mL)
15-Acetyldeoxynivalenol	6
15-Hydroxyculmorin	2
15-Hydroxyculmoron	12
15-Methyl_epi_Fumiquinazolin A	n.a.
15-Monoacetoxyscirpenol	0.8
16-Ketoaspergillimide	0.28
2-Chlorunguinol	0.032
3-Acetyldeoxynivalenol	0.6
3-Nitropropionic acid	0.4
3-O-Methylvirdicatin	0.08
4-Hydroxyalternariol	1
4-Monoacetoxyscirpenol	n.a.
5-Hydroxyculmorin	20
5-Methylmellein	3
7-Hydroxypestalotin	0.2
A 23187	n.a.
AAL TA Toxin	2.4
Abscisic acid	0.8
Acetyldeoxypentahydroxyscirpenol	n.a.
Acetylpentahydroxyscirpenol	n.a.
Aflatoxicol	1
Aflatoxin B1	0.12
Aflatoxin B2	0.2
Aflatoxin G1	0.16
Aflatoxin G2	0.4
Aflatoxin M1	0.2
Aflatoxin M2	n.a.
Aflatoxin P1	1.2
Aflatrem	n.a.
Aflavarin	n.a.
Agistatin B	4
Agistatin D	2.8
Agistatin E	1.32
Agroclavine	0.12
Aigualomycin D	1
Alamethicin F30	0.4
alpha-Zearalenol	0.4
alpha-ZOL-Glucosid	1.2
Alteichin	n.a.
Altenuene	0.28
Altenusin	32
Alternarian acid	40
Alternariol	0.2
Alternariol-3-glucoside	0.4
Alternariol-9-glucoside	0.4
Alternariolmethylether	0.016
Alternariolmethylether-glucoside	4
Altersetin	0.2
Altersolanol	12
Altertoxin-I	0.4

Altertoxin II	n.a.
Amidepsin B	0.6
Aminooctadecyldecan-3-ol	2.4
Amoxycillin	n.a.
Amphotericin	n.a.
Anacin	3.2
Andrastin A	0.08
Andrastin B	0.2
Andrastin C	n.a.
Andrastin D	n.a.
Anisomycin	0.08
Anomalin A	n.a.
Antibiotic F 1849 A	0.4
Antibiotic L 696474	8
Antibiotic PF 1052	0.8
Apicidin	0.06
Ascochlorin	0.08
Ascomycin	1.2
Asparason A	n.a.
Aspercolorin	0.4
Asperfuran	8
Aspergamide A	1
Aspergillicin Derivat	0.4
Aspergillin PZ	n.a.
Aspergillimide	0.08
Asperglauccide	0.04
Asperlactone	0.016
Asperloxin A	0.4
Asperphenamate	0.02
Asperthecin	n.a.
Aspinolid B	0.32
Aspinonene	n.a.
Aspochalasin C	0.8
Aspochalasin D	0.8
Aspochalasin I	2
Aspochalasin J	2
Aspterric acid	4
Aspyrone	16
Asterric acid	1.6
Asteltoxin	1
Atpenin A5	0.028
Atroventinmethylether	0.6
Aurantiamin A	0.32
Auranticin A	2
Aurasperon B	n.a.
Aurasperon C	n.a.
Aurasperon G	n.a.
Aureobasidin A	1.6
Aurofusarin	1.2
Austalide A	0.4
Austalide B	0.4
Austalide D	1
Austalide F	1
Austamide	0.32
Austdiol	n.a.
Austocystin A	0.8
Austocystin D	1
Austocystin I	1.6

Avenacein Y	20
Averantin	0.02
Averantinmethylether	0.032
Averufanin	0.032
Averufin	0.032
Averufin-Derivat	0.2
Bacitracin	n.a.
Bafilomycin A1	0.16
Barceloneic acid A	3.2
Bassianolide	0.2
Beauvericin	0.004
Benzomalvin A	4
Benzomalvin C	1
Berkedrimane B	0.2
Berkeleyacetal B	60
beta-Zearalenol	0.6
beta-ZOL-Glucosid	1.2
Bikaverin	4
Bis(dethio)methylthiogliotoxin	0.4
Bismethylgliotoxin	2
Botryan-Derivat	n.a.
Brasiliamide A	1.2
Brefeldin A	8
Brevianamid F	0.08
Brevicompanine B	0.4
Butenolid	2.8
Butyrolacton I	4
Butyrolacton II	2
Butyrolacton III	1
Calonectrin	2.8
Calphostin C	2
Calyxanthone	4
Cephalochromin	n.a.
Cephalosporin C	n.a.
Cercosporamide	0.1
Cercosporin	6
Cereulide	0.2
Cerulenin	40
Chaetocin	6
Chaetoglobosin A	40
Chaetoglobosin D	40
Chaetomin	8
Chaetoviridin A	0.4
Chanoclavine	0.04
Chetoseminudin	n.a.
Chevalone C	0.12
Chlamydospordiol	0.08
Chlamydosporol	0.4
Chloramphenicol	0.06
Chlorocitreorosein	1
Chloronectrin	0.08
Chlortetracyclin	n.a.
Chrodrimanin	4
Chromomycin A3	n.a.
Chrysogine	0.2
Chrysophanol	4
Citreorosein	0.32
Citreoviridin A	2

Citreoviridin C	n.a.
Citreoviridinol	n.a.
Citrinin	0.08
Citromycetin	10
CJ 21058	0.06
Cladosporin	0.8
Clonostachydiol	0.4
CNM 115443	0.4
Cochliodinol	n.a.
Cochlioquinone A	n.a.
Communsein B	0.2
Cordycepin	1
Culmorin	4
Curvularin	0.4
Curvulin	0.12
Cycloaspeptide A	3.2
Cycloechinulin	0.12
Cycloheximide	1
Cyclo (L-Leu-L-Pro)	0.32
Cyclo (L-Pro-L-Tyr)	0.4
Cyclo (L-Pro-L-Val)	0.32
Cyclopenin	0.08
Cyclopenol	0.8
Cyclopeptine	0.2
Cyclopiazonic acid	6
Cyclosporin A	2
Cyclosporin B	2
Cyclosporin C	2
Cyclosporin D	0.2
Cyclosporin H	0.4
Cylindrol B	0.012
Cylindrocarpon A	0.008
Cytochalasin D	0.4
Cytochalasin E	1.2
Cytochalasin H	8
Cytochalasin J	0.4
Daunorubicin	6
Deacetylneosolaniol	6
Decalonectrin	2.8
Decarestrictin	8
Dechlorogriseofulvin	0.6
Dechloroisochromophilon IV	0.6
Deepoxy-deoxynivalenol	1
Dehydroaustinol	1
Dehydrocurvularin	0.8
Dehydrocyclopeptine	0.8
Dehydrogriseofulvin	0.2
Demethylasteltoxin	3.2
Demethylsulochrin	0.32
Deoxyaltersolaniol A	n.a.
Deoxybrevianamid E	4
Deoxyfusapyrone	0.4
Deoxynivalenol	0.6
Deoxynivalenol-3-Glucoside	0.4
DON-Glutathion	n.a.
Deoxynivalenol-3-Sulfate	n.a.
Deoxynivalenol-15-Sulfate	n.a.
Deoxypentahydroxyscirpenol	n.a.

Deoxytryptoquialanine	n.a.
Deoxytryptoquivaline A	1
Desoxypaxillin	n.a.
Desoxyverrucosidin	n.a.
Destruxin A	0.2
Destruxin B	0.4
Destruxin CHL	3.2
Destruxin D	0.4
Destruxin Ed	4
Dethiosecoemestrin	n.a.
Diacetoxyscirpenol	0.2
Dichlormethylasterric acid	0.4
Dechloronornidulin	0.032
Dihydrobotrydial	n.a.
Dihydrochlamydocin	0.28
Dihydrocitrinone	1
Dihydroergosine	0.08
Dihydroergotamine	0.32
Dihydrogriseofulvin	n.a.
Dihydroinfectopyron	1.6
Dihydrolysergol	0.2
Dihydrosterigmatocystin	n.a.
Dihydrotrichotetronine	4
Dihydroxycalonectrin	n.a.
Dihydroxymellein	1
Dimethoxymethylgrisantrion	n.a.
Dihydroxy-ZON-Mrethylether	n.a.
Dinactin	n.a.
Diplodiatoxin	4
Doxorubicin	2
Doxycyclin	n.a.
Drimane 6	12
Drimane 8	0.8
Elymoclavine	0.2
Elymoclavine-Fructoside	0.6
Emindole SA	n.a.
Emodin	0.028
Enniatin A	0.008
Enniatin A1	0.016
Enniatin B	0.012
Enniatin B1	0.02
Enniatin B2	0.02
Enniatin B3	0.0016
epi-Equisetin	0.12
Epoxyagroclavin	n.a.
Epoxycytochalasin C	0.4
Epoxycytochalasin D	0.4
Equisetin	0.12
Eremofortine A	2
Eremofortine B	20
Ergine	0.04
Ergocornine	0.32
Ergocorninine	0.08
Ergocristam	n.a.
Ergocristinam	n.a.
Ergocristine	0.6
Ergocristinine	0.08
Ergocryptine	0.32

Ergocryptinine	0.2
Ergometrine	0.6
Ergometrinine	0.04
Ergosinine	0.12
Ergosine	0.4
Ergotaminine	0.08
Ergotamine	0.32
Ergovaline	n.a.
Erythromycin	n.a.
F01 1358-A	0.4
Fallacinol	0.1
Fellutannine A	0.32
Fellutannine B	1.6
Festuclavine	0.4
FK 506	1
FK 9775 A	n.a.
FK 9775 B	n.a.
Flavipucin	0.2
Flavoglaucin	0.12
Fonsecin	n.a.
FS4	n.a.
Fulvic acid	3
Fumagillin	8
Fumifungin	16
Fumigaclavine C	1.2
Fumigaclavine A	0.16
Fumiquinazolin A	0.8
Fumiquinazolin D	0.8
Fumiquinazolin E	n.a.
Fumiquinazolin F	8
Fumiquinazolin I	n.a.
Fumiquinazolin Derivat	8
Fumitremorgin A	1
Fumitremorgin B	n.a.
Fumitremorgin C	1
Fumonisin A1	n.a.
Fumonisin A1 precursor	1
Fumonisin A2	n.a.
Fumonisin B1	1.6
Fumonisin B2	1.2
Fumonisin B3	1.2
Fumonisin B4	n.a.
Fumonisin B6	n.a.
Fusaproliferin	20
Fusarenon-X	1.6
Fusapyrone	0.4
Fusaric acid	8
Fusarin C	2.4
Fusarinolic acid	n.a.
Fusarielin A	20
Fusidic acid	0
Geldanamycin	0.16
Geodin	2
Geodin hydrate	4
Gibberellic acid	8
Gibepyron D	6
Gliocladic acid	3.2
Gliotoxin	4

Glyantrypine	0.32
Gregatin B	n.a.
Griseofulvin	0.6
Griseophenone B	1
Griseophenone C	0.12
Harzianopyridone	0.28
Harzianum A	2
HC-Toxin	0.4
Helvolic acid	3.2
Helvolinic acid	n.a.
Heptaibin	10
Heptelidic acid	1.2
Herquline A	0.08
HT-2 Glucoside	0.8
HT-2 Toxin	1.6
hydrolysed Fumonisin B1	0.8
hydrolysed Nidulin	0
Hydroxycarnequinazoline A	0.6
Hydroxycurvularin	0.6
Hydroxyroquefortine C	n.a.
Hydroxysydonic acid	0.8
Hypothemycin	2
Ilicicolin A	0.08
Ilicicolin B	0.16
Ilicicolin C	0.32
Ilicicolin E	0.08
Infectopyron	4
Infectopyron-Derivat	8
Integracin A	0.04
Integracin B	0.04
Ionomycin	1.6
Irgasan	40
Isochromophilon III	10
Isochromophilon IV	0.32
Isochromophilon VI	0.6
Isochromophilon IX	0.6
Isofusidienol	1
Isokotanin B	0.8
Isorhodoptilometrin	0.032
Josamycin	n.a.
K252a	1.6
K252b	8
K-76 Derivative 4	0.32
Kipuksain B	1
Kipuksain D	3.2
KO 143	8
Kojic acid	8
Koninginin D	12
Koninginin E	1
Linamarin	1.2
Lincomycin	n.a.
LL-Z 1272e	0.032
Lolitrem B	n.a.
Lolitrem N	n.a.
Lotaustralin	0.16
Luteoskyrin	n.a.
Luteusin A	1
Lysergol	0.4

Macrosphaelide A	0.12
Macrosporin	0.02
Malformin A	0.4
Malformin A2	0.4
Malformin C	0.08
Marcfortine A	0.08
Marcfortine B	n.a.
Marcfortine C	n.a.
Meleagrin	3
Meleagrin Derivat	1.2
Methoxycurvularin	1
Methoxysterigmatocystin	0.4
Methylasterric acid	0.08
Methylsulochrin	0.12
Methysergid	0.2
Mevastatin	1.2
Mevinolin	0.4
Mithramycin C	n.a.
Mitomycin	n.a.
Monactin	0.12
Moniliformin	0.8
Monocerin	0.2
Monomethylcurvulin	1.2
Mycophenolic acid	0.12
Mycophenolic acid IV	0.4
Myriocin	1.6
N-Benzoyl-Phenylalanin	0.4
Neoechinulin A	0.32
Neosolaniol	0.8
Neoxaline	0.2
NG 012	0.6
Nidulin	0.12
Nidurufin	0.08
Nigericin	0.08
NIgragillin	n.a.
Nivalenol	0.6
Nivalenol-Glucosid	1.6
Nonactin	0.012
Norlichexanthone	0.12
Nornidulin	0.04
Norsolorinic acid	0.4
Nortryptoquialanine	n.a.
Notoamide E	0.4
Notoamide Derivat	0.4
NP 19199	3.2
NP 12318	0.4
NT-2 Toxin	3.2
NX-1	n.a.
NX-2	n.a.
NX-3	n.a.
Ochratoxin A	0.2
Ochratoxin alpha	4
Ochratoxin B	0.8
Ochratoxin C	0.12
Ochrephilone	0.2
Okaramine D	0.32
Oligomycin A	4
Oligomycin B	2.4

O-Methylsterigmatocystin	0.12
Oosporin	0
Ophiobolin A	0.8
Ophiobolin B	3.4
Orsellinic acid	20
oxidized Elymoclavin	1
oxidized Luol	4
Oxalicine	3
Oxaline	0.2
Oxaspirodion	40
Oxytetracyclin	n.a.
Papyracillic acid	2.8
Paracelsin A	n.a.
Paracelsin B	n.a.
Paraherquamide A	0.4
Paraherquamide E	0.2
Paspalic acid	200
Paspalin	n.a.
Paspalinin	n.a.
Paspalitrem A	n.a.
Paspalitrem B	n.a.
Patulin	2.4
Paxillin	4
Penicillazaphilone B	0.3
Penicillic acid	2
Penicillide	0.48
Penicillin G	0.2
Penicillin V	n.a.
Penigequinolone A	0.12
Penitrem A	0.4
Pennigritrem A	20
Pentahydroxyscirpenol	4
Pentoxyfylline	0.08
Pestalotin	0.2
Phenopyrrozin	1.2
Phomalactone	0.4
Phomalone	0.12
Phomopsin A	0.4
Phomopsin B	n.a.
Phomopsolide B	0.4
Physcion	3.2
Piscarinin A	1
Porritoxinol	3.2
Prehelminthosporol	n.a.
Prehelminthosporollacton	n.a.
Prelaptin	0.08
PR Toxin	n.a.
Pseurotin A	4
Pseurotin D	n.a.
Puromycin	0.4
Purpactin A	0.4
Purpuride	0.12
Pyranonigrin	28
Pyrenophorol	1.2
Pyrenocin A	1.2
Pyripyropene A	2.8
Pyripyropene B	n.a.
Pyripyropene D	0.4

Pyrophen	0.4
Quadrone	2
Questiomycin A	0.8
Quinadoline A	1
Quinadoline B	4
Quinolactacin A	0.04
Quinocitrinine A	0.04
Radicicol	0.6
Radiclonic acid	1
Rapamycin	12
Rasfonin	3.2
Roquefortine C	1
Roquefortine D	0.4
Roquefortine E	0.2
Roridin A	0.6
Roridin L-2	n.a.
Rubellin D	0.28
Rubratoxin A	n.a.
Rubrofusarin	2
Rugulosin	0.2
Rugulosuvine	0.12
Rugulotrosin	12
Rugulovasine A	0.28
Sambucinol	8
Satratoxin F	n.a.
Satratoxin G	n.a.
Satratoxin H	n.a.
Scalusamid A	0.6
Sclerotioramin	1.2
Sclerotiorin	1
Sch 725680	n.a.
Secalonic acid D	4
Secoemestrin C Derivat	10
Seco-Sterigmatocystin	0.12
semi Vioxanthin	0.8
semi Xanthomegnin	n.a.
Siccanin	0.2
Siccanol	n.a.
Setusosin	1.2
Skyrin	0.2
S-Methyl-deoxynivalenol	n.a.
Sorbicillactone	400
Sphingofungin B	32
Sphingofungin D	0.32
Spiramycin	n.a.
Spirodihydrobenzofuranlactam IV	0.8
Sporogen AO I	4
Stachybotramide	2
Stachybotrylactam	0.4
Staurosporin	4
Stemphylperylenol	16
Sterigmatocystin	0.16
Sulochrin	0.6
Sydonic acid	0.4
Sydowinin A	1
Synazerol	n.a.
T2-Glucoside	n.a.
T2-Tetraol	1.4

T2-Toxin	0.4
T2-Triol	6
Taxol	n.a.
Tenellin	12
Tensidol B	n.a.
Tentoxin	0.04
Tenuazonic acid	4
Ternatin	0.2
Terpendole C	n.a.
Terpendole E	n.a.
Terphenyllin	0.6
Terrecyclic acid	20
Terrein	4
Terretonin	3.2
Terretonin F Derivat	0.6
Territrem B	1.2
Tetracycline	2
Tetrahydrobostrycin	0.32
Thielavin B	0.2
Thiolutin	0.6
TR-2 Toxin	n.a.
TriacetoxyDON	0.6
Trichalasin B	1
Trichodermamide C	0.32
Trichodermin	2
Trichodimerol	1
Trichostatin A	0.8
Trichothecolone	n.a.
Trichothecin	n.a.
Trypacidin	n.a.
Tryprostatin A	0.6
Tryprostatin B	n.a.
Tryptophol	4
Tryptoquialanine	n.a.
Tryptoquialanine-Derivat	0.12
Tryptoquialanone	0
Tryptoquivaline A	0.4
Tryptoquivaline F	3.2
Tryptoquivaline G	0.32
Tylosin	n.a.
Unguinol	0.08
Unugisin E	0.6
Usnic acid	0.016
Ustiloxin A	n.a.
Ustiloxin B	n.a.
Ustiloxin D	n.a.
Ustusol A	0.32
Valinomycin	2
Vancomycin	n.a.
Vermistatin	0.1
Verrucarin A	n.a.
Verrucarin J	n.a.
Verrucarol	n.a.
Verrucofortine	0.012
Verrucosidin	n.a.
Verruculogen	20
Verruculotoxin	0.12
Versicolorin A	0.12

Versicolorin C	0.12
Verticillin A	10
Violaceic acid	0.6
Violaceol I	4
Violaceol II	4
Viomellein	80
Vioxanthin	n.a.
Viridicatin	0.6
Viridicatol	1.2
Viridicatum toxin	8
Viridol	0.8
WIN 64821	0.4
WIN 68577	4
Wortmannin	2
Xanthocillin	4
Xanthomegnin	4
Xanthotoxin	0.16
Yaequinolone J2	n.a.
Zaragozic acid A	n.a.
Zearalenone	0.06
Zearalenone-16-Glucoside	n.a.
Zearalenone-4-Glucoside	n.a.
Zinndiol	0.32
Zinniamide	0.4
Zinniol	6