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Lina Smolskaite

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Lina Smolskaite. Rare forest and coastal-dune mushrooms: evaluation of antioxidant and biological properties. Agricultural sciences. Institut National Polytechnique de Toulouse - INPT; Kauno technologijos universitetas (Kaunas, Lituanie), 2016. English. NNT : 2016INPT0101 . tel-04300503

HAL Id: tel-04300503

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Université
de Toulouse

THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

Institut National Polytechnique de Toulouse (Toulouse INP)

Discipline ou spécialité :

Sciences des Agroressources

Présentée et soutenue par :

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le lundi 7 novembre 2016

Titre :

Champignons oubliés sylvestres et de dunes : étude de leurs propriétés
anti-oxydantes et biologiques

École doctorale :

Sciences de la Matière (SDM)

Unité de recherche :

Laboratoire de Chimie Agro-Industrielle (LCA)

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ABBREVIATIONS

ABTS⁺ – 2,20-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt;
FRAP – ferric reducing antioxidant power;
ASE – ‘antioxidant score’ of extract;
ASM – antioxidant scores of mushrooms;
C – cyclohexane;
CC50 – cytotoxic concentration of the substance to cause death to 50% of viable cells in the host;
CFU – colony forming unit per mL;
CIU – comparative integrated units;
COSY – correlation spectroscopy;
D – dichloromethane;
DMSO – deuterated dimethylsulfoxide;
DPPH[•] – 2,2-*di*-phenyl-1-picrylhydrazyl hydrate radical;
EDW – dry extract weight;
EC₅₀ – effective concentration 50%;
ESI – electrospray ionization;
EX – exsheatment;
EY – extract yields;
FCS – fetal calf serum;
FID – flame ionization detector;
FL – fluorescein;
GAE – gallic acid equivalent;
GLM – general linear model;
HAT – hydrogen atom transfer;
HMBC – heteronuclear multiple-bond correlation spectroscopy;
HPLC – high performance liquid chromatography;
HSQC – heteronuclear single-quantum correlation spectroscopy;
IC50 – inhibition concentration 50%;
M – methanol;
MIC – minimal inhibitory concentration;
MS – mass spectrometry;
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide;
N.D. – not detected;
N.T. – not tested;
NMR – nuclear magnetic resonance spectroscopy;
ORAC – oxygen radical absorbance capacity;
PBS – phosphate buffered saline;
PDA – photo diode array detector;
Q-TOF – quadrupole time-of-flight;

R – correlation coefficient;
R_f – retention factor;
RPM – rotation per minute;
RPMI – Roswell Park Memorial Institute;
RSC – radical scavenging capacity;
R_t – retention time;
SD – standard deviation;
SET – single electron transfer;
SI – selectivity index;
SQD – simple quadrupole detector;
TE – Trolox equivalents;
TEAC – trolox antioxidant activity coefficient;
TLC – thin layer chromatography;
TPC – total phenolic content;
TPTZ – 2,4,6-tripyridyl-s-triazine;
U/mL – units per volume;
UPLC – ultra performance liquid chromatography;
UV – ultraviolet;
V/V – volume to volume;

INTRODUCTION

Due to the metabolic diversity, ecological variety, significant role in nature, complicated web of life cycles, fungi have attracted the attention of various scientists mycologists, chemists, biologists, pharmacists in multiple ways (Tkacz, 2004). The amounts of mushrooms consumption has been increasing in recent years, and they remain commercially important sources for the producers of speciality foods (Nowacka et al., 2014). Also scientific expertise is increasingly being applied to help achieve the primary development goals, which include poverty alleviation and sustainable use of natural resources. Real progress has been and continues to be made in the roles that mushrooms conduce towards these goals (Boa, 2004).

In the past years, many new secondary metabolites with diversified chemical structures and various interesting bioactivities from higher fungi have been isolated. These molecules are more likely to provide lead compounds for new substances that can improve biological body functions or medicinal discovery, like chemopreventive agents possessing the bioactivity of immunomodulatory, anticancer, etc. However, numerous challenges of bioactive molecules from mushrooms are encountered including bioseparation, identification, biosynthetic metabolism or screening model issues. Researchers have constantly searched new active substances from mushrooms, however the commercial products are yet very limited and mainly from field cultivation, which is obviously a time-consuming and labor-intensive process (Zhong and Xiao, 2009). Strategies to enhancing new secondary metabolite existence as useful natural products in industry the wild unknown, underinvestigated mushroom species could be an extensive source. Moreover among the known species the relative amount of well investigated mushrooms is very low. This fact together with the information about the great potential of microscopic fungi for production of bioactive metabolites, the experience in ethnomedicinal use of mushrooms, the ecologic need for fungi to produce bioactive secondary metabolites and the improved opportunities for genetic, pharmacological and chemical analysis let us believe that mushrooms have a great potential for successful bioprospecting (Lindequist, Niedermeyer and Julich, 2005). A large, untapped, and chemical diversity resource of bioactive metabolites from wild macrofungi provide more and more opportunities for finding new lead structures for medicinal, nutritional, functional, therapeutical, phytochemical chemistry, and a new era of higher fungi secondary metabolite research has appeared.

According to folk mycologist knowledge less known wild mushrooms grown in Midi-Pyrénées region, France, might be a good source of bioactive constituents. Scarce information on presence of natural secondary metabolites and their properties such as antioxidants, antimicrobials and other food protecting and/or health promoting compounds on selected wild mushroom species have not performed until now. On this basis main hypothesis maintain, that wild mushrooms species may contain valuable natural bioactive molecules with multifunctional properties used for various food and medicinal applications, such as ingredients of functional foods and nutraceuticals. Meanwhile, hypothesis needed to be investigated, finding promising source of natural bioactive molecules and carrying out detail investigations of

promising ones, on the optimization of detection techniques on biochemical studies towards safety of newly discovered sources, and the bioavailability of active molecules.

The aim of the thesis

The main aim of this study was to investigate antioxidant, antimicrobial and some other bioactivities of the selected underinvestigated wild mushroom species for obtaining scientific information, which is required for their valorization as a source for human nutrition and production of functional ingredients.

The objectives of this study:

1. To fractionate dried mushroom material using increasing polarity solvents and to determine extract yields.
2. To evaluate antioxidant activity of mushroom fractions by using several *in vitro* assays.
3. To evaluate antimicrobial activity of mushroom fractions by using various microorganisms.
4. To evaluate cytotoxicity properties of the selected wild mushrooms species.
5. To isolate and purify secondary metabolites from the selected mushrooms species using various chromatographic methods.
6. To elucidate the structures of purified metabolites using spectroscopic techniques such as ESI-MS, ESI-MS/MS, ^1H NMR, ^{13}C NMR, HMBC, COSY, HSQC.
7. To evaluate antioxidant and cytotoxicity activity of the main secondary metabolites, isolated from selected mushroom species.
8. To examine the influence of selected mushrooms extracts collected at different mushroom harvesting periods and their isolated compounds inhibitory activity properties against gastrointestinal nematodes *in vitro*.

Scientific novelty

Scientific novelty of the research performed may be summarized by the following main points:

- Systematic and comprehensive evaluation of antioxidant, antimicrobial and cytotoxic properties of various mushroom species from Midi-Pyrénées region of France has been performed for the first time;
- Some compounds isolated from the analysed mushroom species have been reported for the first time;
- The effects of identified in some mushroom species compounds against gastrointestinal nematodes, as well as their antioxidant and cytotoxic activity have been evaluated for the first time;
- The extracts from selected mushroom species have been reported as antihelmintic agents for the first time;

Practical significance

Wider exploration of underinvestigated bioresources, including various mushroom species, requires sound scientific evidence about their composition and properties. From this point of view comprehensive and systematic evaluation of antioxidant, antimicrobial and some other properties of wild Midi-Pyrénées mushroom species, may be considered as an important step for practical valorization of these species as a sources of natural functional ingredients to be applied in nutraceuticals, functional foods and other applications.

More specifically, the new knowledge obtained on hispidin, hispolon and inonotic acid methyl ester, the compounds which were identified in mushroom extracts and which have been characterized for their cytotoxic and antioxidant activity may be practically useful for industries searching for new structures to be incorporated into food, nutraceutical, pharmaceutical and cosmetic products as efficient bioactive natural ingredients.

Finally, the *in vitro* assay results confirmed the potential activity of selected mushrooms species and the compounds isolated thereof to inhibit gastrointestinal nematodes. This knowledge may be practically applied for developing new effective preparations possessing anthelmintic activities for increasing resistance to worm populations in ruminants. For instance, mushroom extracts might a promising candidate as an alternative of synthetic drugs causing their residues in the animal raw material, which is important characteristic in the production of organic livestock.

The key points of the thesis

1. Selected wild mushroom species contain valuable bioactive constituents, such as antioxidants, antimicrobial and cytotoxic agents, while proper selection of solvents enables to obtain the extracts with the highest concentration of bioactive compounds which might be a valuable source of natural products for various applications.
2. The selected mushroom genus *P. schweinitzii* and *I.hispidus* have the potential *in vitro* activity against gastrointestinal nematodes; however depending on mushroom harvesting period, anthelmintic activity may change, in terms of active constituents concentration accumulated in selected genus.

Structure and content of the dissertation

The dissertation is written in English. It consist of acknowledgements, list of abbreviations, introduction, literature review, materials and methods, results and discussion, conclusions, list of references, annexes, list of publications relevant to results of thesis. The dissertation has 137 pages in total, results presented in 9 tables and 41 figures. The list of references includes 242 bibliographic sources.

1. LITERATURE REVIEW

1.1. HISTORY, DIVERSITY AND IMPORTANCE OF MUSHROOMS

1.1.1. General characterization and definition of mushrooms

Historically, mushrooms were classified as lower plants. Due to the relatively simple anatomical structural attributes such as lack of true roots, true stems, true leaves, true flowers and true seeds. The existence of a cell wall linked them to plants rather than to animals. Improved technologies and scientist found that mushrooms biota have their own characteristics, together with other fungi, which are sufficiently and significantly particular. The chitin cell wall possession is different in composition from plants (cellulose or hemicellulose) and a mode of nutrition is heterotrophic but, unlike animals, is absorptive (osmotrophic) rather than digestive (Miles and Chang, 2004). Therefore mushrooms have been placed in a kingdom, called Myceteae separate from pants and animal kingdom. Mushrooms can produce a wide range of enzymes that degrade complex substrates (agricultural, industrial and forest waste products) and forming their own nutrition with soluble substances. Mushrooms can be classified into three ecological groups: saprophytic (grows on complex organic material, found in dead or living tissues), parasitic (deriving their food substances from living plants) and mycorizzal or mutualistic symbiotic (living in association with roots of vascular plants) (Stamets).

The word mushroom has been used in variety of ways at different times and may mean different things to different people or countries. In a broad sense the term mushroom include all the larger fungi, or all fungi with stem and caps, which are edible and/or have medicinal value (Miles and Chang, 2004). More than twenty years ago scientist (Miles and Chang, 1997) described properly: “mushroom is macrofungus with a distinctive fruiting body, which can be either hypogeous (under ground) or epigeous (above ground), large enough to be seen with the naked eye and to be picked by hand”. From a taxonomic point of view, mainly basidiomycetes but also some species of ascomycetes belong to mushrooms. *Basidiomycota* mostly contain macrofungi, including mushrooms and toadstools, bracket fungi and puffballs, although 30% of its species are microscopic. Some “larger fungi” cup-fungi, morels and truffles belong to *Ascomycota* (Senn-Irlet et al., 2007).

1.1.2. History, traditions and importance of mushrooms

Mushrooms have been a part of the fungal variety since for around 300 million years ahead. First records of fungi have been known from the old fossil in the time as the Silurian period, 408 to 438 million years ago – in the Paleozoic era. Fungal diversity, included Basidiomycetes and Ascomycetes with some fruiting bodies, had increased by the Pennsylvanian period since 286 to 320 million years ago. Probably prehistoric native people used wild collected mushrooms as food and perhaps as medicinal purpose (Miles and Chang, 2004). As the evidence, mushroom has been represented in Tassili image from a cave dating back 5000 years B.C. Approximately 1700 years later first primates have been used Brich Polypores (*Piptoporus betulinus*), tinder fungus (*Fomes fomentarius*) and mushroom that may

have magical-spiritual significances. Polypores can be used as spunk for starting fires and medicine for treating injury. As well as, tea prepared by boiling these mushrooms may help as antibacterial and tonic attributes. This data received from well-preserved remains of man named “Iceman” or “Oetzi” found in 1991 (Stamets). Other archaeological record reveals edible mushroom species associated with people living 13 000 years ago in Chile. Although it is in China where the eating of wild fungi is first reliably noted, with a long history of use for their health promotion benefits, reach several hundred years before the birth of Christ. Edible fungi were collected from woodlands in ancient Greek and Roman times and highly valued as delicacy, though more by high-ranking people than by farmers. Caesar's mushroom (*Amanita caesarea*) is a good example of an ancient tradition and memorable history that still exists in many parts of Italy, embracing a diversity of edible species consumed today by truffles (*Tuber spp.*) and porcini (*Boletus edulis*) (Boa, 2004). The early civilizations of the Greeks, Egyptians, Romans, Chinese and Mexicans appreciated mushrooms as a delicacy, knew something about their medical properties, and used them in religious ceremonies also as devious purposes. Claudius II and Pope Clement VII were both killed by enemies who poisoned them with deadly *Amanita*. Further, according to the legend, Buddha died from mushroom that grew underground. Less powerful, psychoactive mushrooms have been used in religious ceremonies and humans believed their spiritual changes, till Gordon Wasson, Albert Hofmann and Carl Ruck discovered shamanic use of psilocybin-containing mushrooms (Stamets). This strong emphasis on hallucinogenic mushrooms has unlocked academic interest of wild edible fungi. During the last twenty to thirty years researchers have substantially increased our knowledge of mushroom world (Boa, 2004). In present years reports on the chemistry, and the nutritional and functional properties of mushroom have been overwhelmed. More than 300 articles related to mushrooms have been published in the last two decades (Rathee et al., 2012) and the interest on mushrooms is still growing.

The Chinese have for centuries valued many species, not only for food and highly desirable taste but also for their, tonic and medicinal properties. These values and traditions are as strong nowadays as they were centuries ago. As well as the huge assortment of mushrooms collected from forests and fields and marketed widely. In China 600-1000 a.d. were first mushrooms *Auricularia auricula*, *Flammulina velutipes*, *Lentinula edodes* cultivated on wood logs, today this country are leading exporter of cultured mushrooms (Boa, 2004, Miles and Chang, 2004). For some regions in China where the rate of mushrooms consumption reaches even 20-24kg fresh product per capita annually, which is relatively higher than in Europe e.g. Sweden 1kg, Czech Republic 7-10kg per capita annually (Zhang et al., 2010). Yunnan province of China has an area of 390,000 km², which is a specific region abundant in wild-grown mushrooms and over 880 species are identified as edible. In this region about 80% of the edible species identified in China and around 40% in the world (Wang et al., 2014a). China also exports wild edible mushrooms, e.g. the export rate of Yunnan in was 105,000 (2010 year) and 135,000 (2011 year) tones per

year. One of the most valuable mushroom *Tricholoma matsutake* seek more that 80 % of the total export from China, Yunnan (Wang et al., 2014a).

There are approximately 100 mushroom species in Lithuanian forests, however only 15-25 of them are collected as edible mushrooms. The annual exploitable mushroom yield is 15 to 30 kg per hectare, and it is believed that 30-40% of the exploitable harvests are collected. Edible wild mushroom production in Lithuanian forests amounts to 24 000 tones and industrial production to about 8000 tonnes (Olmos, 1999).

The biggest advance in mushroom cultivation came in France about 1600 when *Agaricus bisporus* was cultivated on composted substrate. *A. bisporus* commonly known as champignon or the button mushroom increased rapidly in popularity from early beginning and today is produced in the biggest quantity (Miles and Chang, 2004). Mushroom cultivation fluctuates from year to year, depending on demands, but the production of forest mushrooms is not all predictable. Picking their fruit bodies is a popular pastime and a recreational activity for many people in substantial number of countries.

1.1.3. Diversity, composition and characteristics of mushrooms

Today mushrooms are one of the most species rich and diverse groups of organisms on Earth. Fungi constitute a significant part of terrestrial environment, forming a large share of the species richness and are key-players in ecosystem processes. The kingdom of fungi were long considered as unfamiliar group of organisms, poorly understood and difficult to study due to their largely hidden nature and frequently sporadic and short-lived fruiting body. However, through the research of scientist and contribution of mycologists over the last few decades our knowledge of fungi has significantly increased.

According to (Miles and Chang, 2004) mushrooms can be roughly divided in four categories: freshly and edible mushrooms (e.g. *A. bisporus*); medicinal mushrooms – that have medicinal applications (e.g. *Ganoderma lucidum*), poisonous mushrooms – those that are proved to be or suspected as poisonous (e.g. *Amanita phalloides*), diverse category – which includes a large number of mushrooms whose properties remain less well defined. These may tentatively be grouped together as “other mushrooms”. Some mushrooms fall into more than one category; such as mushrooms that are not only edible, but also have tonic and medicinal properties.

There are estimated at least 1.5 million fungal species worldwide, only 74.000-140.000 species have been described. The number of mushrooms, according to definition given by (Miles and Chang, 1997), are known about 14.000 species, which would account for 10% of the described species (Garibay-Orijel et al., 2009). *Fig. 1.1* describes distribution of known wild edible and medicinal mushrooms species from more than 200 different sources and 110 countries (Boa, 2004).

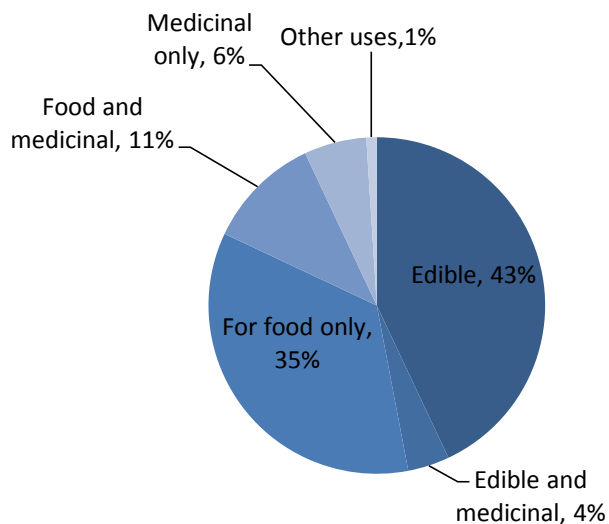


Fig. 1.1 Diversity of wild edible and medicinal mushrooms species. (Note: varieties and subspecies are counted separately. The categories ‘food’ and ‘edible’ are mutually exclusive. To distinguish clearly between use and properties of a species: substantial numbers of edible species lack confirmed use as food)

Furthermore, only 200 species are experimentally grown, 100 economically cultivated, approximately 60 commercially cultivated, and about 10 have reached an industrial scale of production in many countries. The number of poisonous mushrooms is usually reported approximately 1%, but mushrooms with poisonous symptoms is estimated approximately 10% (Miles and Chang, 2004). To have in mind that proportion of described mushrooms among the undiscovered and unexamined mushrooms will be only 5%, which implies 7000 species yet unknown species will be of possible benefit to humankind and environment (Lindequist et al., 2005).

Obviously, mushrooms have been widely used as human food for centuries and have been appreciated for texture and flavour as well as various medicinal and tonic properties, even were used as a textile dye (Velišek and Cejpek, 2011). Mushrooms are considered as healthy food due to high content of proteins usually 12-40% dry matter, the highest content of 48.8% and 51.2% dry matter were observed in *Calocybe gambosa* and *Macrolepiota mastoidea*. The mushroom protein contains all the nine essential (threonine, valine etc.) amino acids required by humans. Mushrooms also contain relatively large amount of carbohydrates approximately 13-70% dry matter (oligosaccharides, chitin, mannitol, glucose, glycogen etc.) and fibers which is a group of indigestible carbohydrates approximately 4-30% dry matter (Barros et al., 2008, Manzi et al., 2004). In addition, they contain significant amount of vitamins, namely thiamine, riboflavin, niacin, ascorbic acid, tocopherol and vitamin D, as well as minerals (K, Ca Mg, Fe) (Kalač, 2009). They are also valuable foods due to low-calories and low-fat content approximately 2-8%. The differences between polyunsaturated fatty acids and

saturated fatty acids is quite high, constituting more than 75% of total fatty acids, of which oleic and linoleic acids are the most significant, while palmitic acid is the main saturated fatty acid (Ribeiro et al., 2009).

In addition to their nutritional value, taste and aroma edible mushrooms usually contain various bioactive molecules. These naturally occurring chemical compounds or complex substances identified in mushrooms have multifunctional properties.

1.2. MUSHROOM AS A SOURCE OF NATURAL BIOACTIVE MOLECULES

Unlike the primary products participating in nutrition and essential metabolic processes inside the plant or fungi, secondary products play a major role in the adaptation to their environment. Higher fungi have formed a special mechanism of metabolism which could produce diversified functional secondary metabolites containing various properties of complex chemical structure and bioactivity during the long-term evolution. An attempt to resist unfavourable survivable environments and finish cell proliferation, differentiation and the entire life cycle to reach the purpose of self-defence and survival, mushrooms simultaneously provides a rich and fascinating resource for new bioactive molecules (Keller, Turner and Bennett, 2005).

Most bioactive molecules are derived from natural sources and named phytochemicals or pharmacologically active compounds. They play an important role in the human health and safety, during each person lifetime and development, besides reducing disease risks. Many companies are actively engaged in finding new bioactive substances that can influence health or diseased status and that can be incorporated in food products. Several researches in the food industry resemble approaches used in the pharmaceutical world, starting with molecular targets that are frequently derived from drug targets (Georgiou, Garssen and Witkamp, 2011). To combat diseases and adverse health conditions treatment approach where medicines and nutrition complement each other, with phytochemicals in cooperation may prove to be the most reliable (Eussen et al., 2011). More than 80% food active compounds and more than 30% drugs are produced from bioactive natural products.

Higher fungi bioactive substances include a wide range of chemical components and can be divided into: secondary metabolites, proteins and high molecular mass polysaccharides, including polysaccharopeptides and proteoglycans (*Fig. 1.2*). Polysaccharide β -glucan are most widely investigated bioactive molecules from mushrooms, possessing antitumor and immunomodulatory properties and are already used in clinical treatment. New proteins (include lignocellulose degrading enzymes, lectins, proteases and protease inhibitors, ribosome-inactivating proteins and hydrophobins) with biological activities can be used in biotechnological processes and for the development of new drugs. Secondary metabolites from mushrooms combine various structures compounds such as polyketides, terpenes, steroids and phenolic compounds (Barros, Baptista and Ferreira, 2007) with diverse and multifunctional biological activities. Studies on

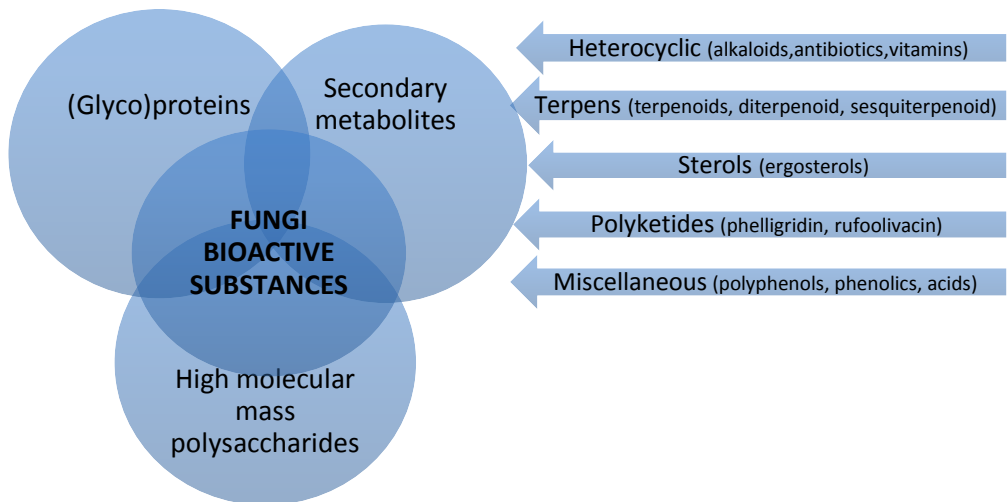


Fig. 1.2 Higer fungi bioactive components

biologically active substances in mushrooms are not only theoretical but also applied. Based on the updated researches, five main aspects may be summarized:

- An incredible diversity of natural bioactive molecules disclosing anti-inflammatory, antitumor, antidiabetic, antibacterial, antiviral and antioxidant activity, also have cardiovascular and liver protective properties (Lindequist et al., 2005, Ferreira et al., 2010, Poucheret, Fons and Rapior, 2006); Moreover, their antiallergic, antiatherogenic, hypoglycemic and hematological properties have been demonstrated (Palacios et al., 2011).
- Mushrooms bioactive compounds are involved in immunomodulating therapies as components that stimulate or inhibit immune system. Polysaccharides such as lentinan, schizophyllan, polysaccharide K (Kerstin), and polysaccharide peptide (PSP) are now approachable in medicine market. For example, lentinan is applied as contributor in cancer immuno-therapy or in parallel to radio- and chemothermotherapy (El Enshasy and Hatti-Kaul, 2013).
- Different mushrooms species can be considered as functional food due to generated bioactive molecules. According to International Life Science Institute (ILSI Europe) the definition which states that “a food can be regarded as functional” if it is satisfactory demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, to improve state of health and reduce the risk of disease. Extensive studies have proved that mushrooms are of value in the prevention and treatment of a number of human diseases.(Guillamón et al., 2010).
- People worldwide rely on plant-based medicines or dietary supplements for their primary health care that can improve biological functions and thereby make people fitter and healthier. These products with one or more active

ingredients have variously been called: vitamins, dietary supplements, phytochemicals, nutraceuticals and nutriceuticals. In 2002, mushroom products (mushroom derivatives from medicinal, edible and wild mushrooms) used mainly for dietary supplements (mushroom nutriceuticals) was assessed to have generated about US\$11 billion and since that time medicinal mushrooms and derived products has increased by between 20-40% annually depending upon the species (Chang and Buswell).

- Mushrooms pigments were used as textile dye before the invention of synthetic dyes (Velišek and Cejpek, 2011) however extracting natural colours from mushrooms has been recently increased (King, 2002). Pigments, that also have bioactive properties might be used as eco-friendly and vast range of vivid coloration agents.

1.2.1. Brief review on the investigated wild mushrooms bioactive molecules

It is evident that mushrooms accumulate a great variety of biologically active organic compounds, secondary metabolites that are not directly involved in primary metabolic processes of growth and development. Recently, researchers have been gradually interested in the chemical composition of mushrooms, although curiosity began in 20th century. The most important discovery of natural active metabolite and antibiotic in medicine was penicillin in 1929, during World War II. Alexander Fleming published his observation on the inhibition of growth of *Staphylococcus aureus* on an agar plate contaminated with micro fungi *Penicillium notatum*, which describes an important path for the development of modern antibiotics (Rishton, 2008). However first fungal bioactive metabolite is mycophenolic acid from *P. glaucoma* that was initially discovered by Bartolomeo Gosio in 1893 as antibiotic agent (Regueira et al., 2011). These antibiotic discoveries laid the foundation of bioactive secondary metabolites research of mushrooms. An increasing attention worldwide for active molecules has continued unabated and thousands of compounds with various properties (antioxidant, antibacterial, antifungal or antitumor etc.) have been investigated and identified in fungi (Pelaez, 2004). In present literature survey of macro fungal metabolite, which reported over 38% (approximately 8600 compounds) from more than 22000 microbial fungi species were described as bioactive substances and these numbers are doubling every 10 years. In addition, around 25000 microbial secondary metabolites, over half of which were derived from higher fungi, need further assay and investigations about bioactive molecules availability. The most frequent producers of bioactive secondary metabolites among macro fungi species belong to the genera *Ganoderma*, *Polyporus* and *Lactarius* (Zhong and Xiao, 2009). Chemical constituents of mushrooms received a lot of attention in the past decades. Numerous further studies have proven the effectiveness of bioactive molecules of investigated mushrooms and resulted in the various applications are summarised in *Table 1.1*.

Many other mushroom species have been extensively studied with respect to their biological activity. *Pleurotus*, *Lentinula*, *Grifola*, *Saparassis*, *Hericium*, *Coriolus*, *Cordyceps*, *Trametes* genus also possessing active substances, were

mainly polysaccharides and proteins represent anti-tumour and immunomodulating effect (Roupas et al., 2012).

Table 1.1 Bioactive molecule and bioactivity and of mushroom genus

Genus name	Compound	Bioactivity	Ref.	
<i>Agaricus sp.</i>	Agaritine derivatives, agaricoline, ergosterol, blazein, phenolic compounds;	Tumor growth reduction, antiviral, antioxidant;	(Itoh, Ito and Hibasami, 2008); (Wisitrassameewong et al., 2012); (Ferreira, Barros and Abreu, 2009).	
<i>Suillus sp.</i>	Suillus, suillumide, pulvinone derivatives, grevilline derivatives, flazin;	Cytotoxic against the human melanoma, antioxidant, anti-inflammatory, anti-fever, anti-ageing, anti-HIV;	(Zhong and Xiao, 2009); (Leon et al., 2008, Yun et al., 2001, Klostermeyer et al., 2000)	
<i>Phellinus sp.</i>	phelligrudin (A- J), phelligrudimer, hispidin, davallialactone;	Antioxidant, antibacterial, anticancer;	(Mo et al., 2004); (Reis et al., 2014, Lee et al., 2008a).	
<i>Paxillus sp.</i>	Paxillamide, derivatives, derivatives;	curtisians ergosterol	Antioxidant, anticancer;	(Gao et al., 2001a, Gao et al., 2004)
<i>Russula sp.</i>	Aristolane, nardosinane, russulanobilines, nigricanice and derivatives;	Anticancer, antimicrobial;	(Vidari, Che and Garlaschelli, 1998, Malagòn et al., 2014, Tan et al., 2004);	
<i>Lactarius sp.</i>	Rufuslactone, derivatives, derivatives, compounds;	lactarane mitissimol phenolic	Antifungal activity, against tumor-causing cells, antioxidant;	(Luo et al., 2005);(Kim et al., 2010, Luo et al., 2006); (Ferreira et al., 2009, Gao et al., 2001b).
<i>Tuber sp.</i>	Tuberoside, cerevisterol ergosterol derivatives.	Antiviral;	(Gao et al., 2001b).	
<i>Leucopaxillus sp.</i>	Cucurbitacins, leucopaxillone (A,B) triterpene;	Anticancer;	(Clericuzio et al., 2004, Clericuzio et al., 2006)	
<i>Ganoderma sp.</i>	Ganomyacin, lucidenic acid N, methyl lucidenate F, lucialdehyde (A-C) prenylated phenols, hesperetin, formometin, biochanin, triterpenoids;	Antibacterial, antioxidant, as agents against cancer, hepatitis, chronic bronchitis, asthma, hemorrhoids, fatigue symptoms;	(Wu, Shi and Kuo, 2001, Mothana et al., 2000); (Gao et al., 2002); (Peng et al., 2015) (Ferreira et al., 2009, Zjawiony, 2004)	
<i>Cortinarius sp.</i>	Unsymmetrical disulfidecortamidine oxide, symmetrical disulfide	Antimicrobial, anticancer, antifungal, antioxidant;	(Nicholas, Blunt and Munro, 2001, Teichert et al., 2008)	

Genus name	Compound	Bioactivity	Ref.
	cortamidine oxide, quinolone, isocarbostryl alkaloid, polyketide derivatives;		(Bai et al., 2013)
<i>Polyporus sp.</i>	Biformin, polyporusterones A-G, ergosterol derivatives, hispolon, hispidin.	Antimicrobial, cytotoxic activity against leukemia, antiviral;	(Zjawiony, 2004, Awadh Ali et al., 2003)

Most biologically active metabolites from mushrooms possess the drug-like characteristics of chemical structure, which can act as major natural compounds factories for new drug discovery. The drug-likeness of secondary metabolites from higher fungi mainly have molecular weight in range of 150–1000 Da and metabolites usually contain C, H, O, and N, even S, P and chlorine group atoms such as Cl, Br, and F. Also chemical structure commonly contains some important functional groups like hydroxyl, carboxyl, carbonyl, amino, etc., which can provide multipharmacophore points and the number of the donor and receptor of hydrogen-bonding conform the rules of drug-likeness. According to Zhong and Xiao (Zhong and Xiao, 2009) secondary bioactive metabolites of fungi can be grouped as heterocyclics, polyketides, sterols, terpenes, and miscellaneous.

1.2.1.1. Heterocyclic compounds in mushrooms

Heterocyclic compounds are defined as constituting cyclic structures with at least two different atoms as member of the ring. Heterocyclics are mostly emerge and compose almost one-third of the total number of known natural organic products, such as antibiotics, vitamins, dyes, alkaloids, pharmaceuticals and etc. The major types of bioactive molecules represented in mushrooms are nitrogen heterocyclics, oxygen heterocyclics, sulfur-heterocyclics and etc (Zhong and Xiao, 2009).

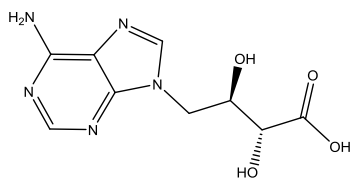


Fig. 1.3 Structural formula of eritadenine

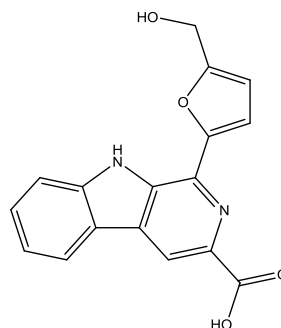


Fig. 1.4 Structural formula of flazin

The shiitake mushroom (*Lentinus edodes*) is traditionally consumed in East Asia, is edible and well established as a medicinal mushroom because it contains several substances that promote health and have antioxidant activity properties (Zhang et al., 2013). The heterocyclic agent responsible for the plasma cholesterol-

reducing effect of shiitake is a secondary metabolite designated eritadenine, (Fig. 1.3) (2R, 3R)-4-(6-Amino-9H-purin-9-yl)-2,3-dihydroxybutanoic acid (Enman et al., 2008).

Other nitrogen-containing heterocycle compound exhibiting a weak anti-HIV-1 activity, namely flazin (Fig. 1.4), 1-[5-(Hydroxymethyl)-2-furyl]-9H- β -carboline-3-carboxylic acid, belonging to β -carboline derivatives, from the fruiting body of *Suillus granulatus* (Zhong and Xiao, 2009). Active secondary metabolites two quinoline (6-Hydroxyquinoline-8-carboxylic acid (1R), 4-Amino-6-hydroxyquinoline-8-carboxylic acid (2R); Fig. 1.5) and isocarbostryl alkaloid (7-Hydroxy-1-oxo-1,2-dihydroisoquinoline-5-carboxylic acid; Fig. 1.5) produce of *Cortinarius subtorus* exhibited antioxidant and antifungal effect (Teichert et al., 2008). 4-hydroxymethyl quinoline (Fig. 1.6) was isolated from the wood-rotting fungi *Trametes versicolor* and *Pycnoporus sanguineus*. This was the first detection in fungi of an unoxidized quinoline nucleus, and it is active against malaria (Abraham and Spassov, 1991) also may have role in plant growth regulation and antifungal activity (Vinale et al., 2010).

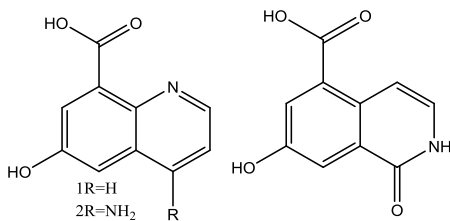


Fig. 1.5 Structural formula of two quinoline (1R, 2R) and isocarbostryl alkaloid

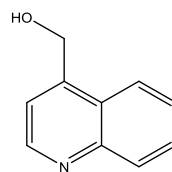


Fig. 1.6 Structural formula of 4-hydroxymethyl quinoline

1.2.1.2. Polyketides in mushrooms

Polyketides are secondary metabolites formed from a polyketone and/or the polyketide chain and containing at minimum one acetate group, comprise a huge class of bioactive molecules with a broad of structural variety. In general, the polyketide chain is formed by subsequent addition of simple carboxylic acids like acetate. Polyketides of higher fungi are the most richest natural organic complex that have been shown to display a wide range of potentially valuable therapeutics due to their antibiotic, anticancer, antifungal, antimicrobial, antioxidant, hypolipidemic and immunosuppressive properties among natural products.

Phellinus igniarius, basidiomycete belonging to the family Polyporaceae has been long used in traditional Chinese medicine, for the treatment of wounds, bellyache, and bloody gonorrhea since ancient times. In recent years scientist has obtained over 20 secondary metabolites with interesting chemical structures and significant bioactivities from *P. igniarius*. Several active polyketides phelligridin C (8,9-dihydroxy-3-[(E)-2-(4-hydroxyphenyl)viny]-1H,6H-pyrano[4,3-c]isochromene-1,6-dione; Fig. 1.7) and phelligridin B (methyl-6-[(E)-2-(3,4-dihydroxyphenyl)-

vinyl]-4-hydroxy-2-oxo-2H-pyran-3-carboxylate; Fig. 1.8) had significant selective cytotoxicity against human lung cancer. Phelligrudin G, containing an unprecedented carbon skeleton, was a unique pyrano derivative ((3-(5-[(E)-2-(3,4-dihydroxyphenyl)ethenyl]-5',6'-dihydroxy-3-oxo-3H-spiro[furan-2,1'-inden]-2'-yl)-8,9-dihydroxy-1H,6H-pyrano[4,3-c]iso-chromene-1,6-dione) which showed not only moderate cytotoxic activities against human cancer cells but also antioxidant activity inhibiting rat liver microsomal lipid peroxidation (Zhong and Xiao, 2009).

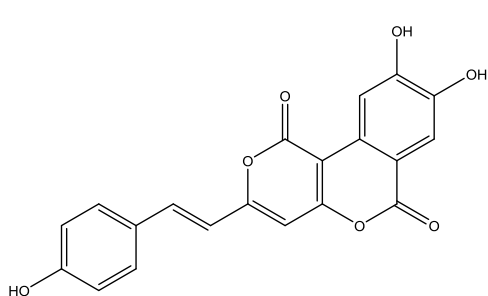


Fig. 1.7 Structure of phelligrudin C

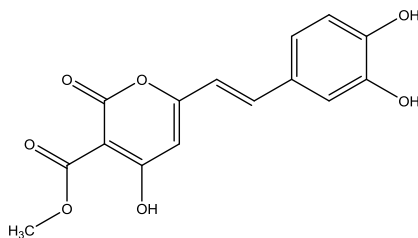


Fig. 1.8 Structure of phelligrudin B

Recently, chemical studies of the ethyl acetate fruiting bodies of *Cortinarius purpurascens* led to the isolation of nine, polyketide-derived, anthraquinone pigments among four of tested natural compounds, rufoolivacin, rufoolivacin C, rufoolivacin D, leucorufoolivacin (Fig. 1.9 and 1.10) exhibited potent DPPH radical-scavenging activity (Bai et al., 2013, Brondz et al., 2007).

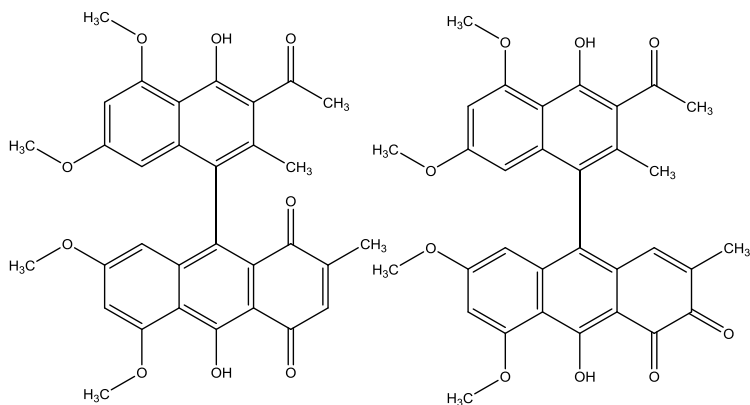


Fig. 1.9 Structure of rufoolivacin, rufoolivacin C

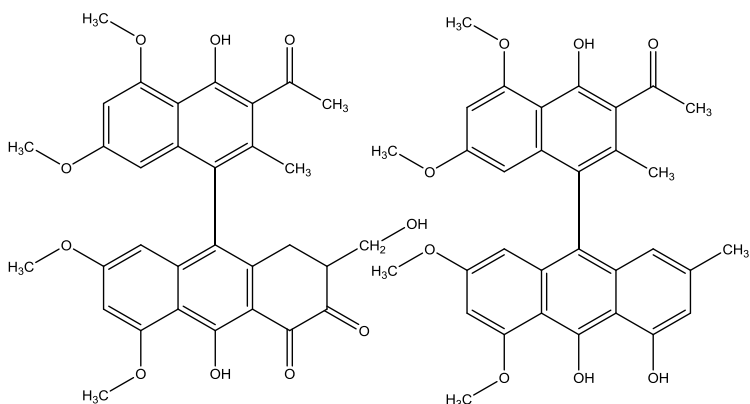


Fig. 1.10 Structure of rufoolivacin D, leucorufoolivacin

1.2.1.3. Sterols in wild mushrooms

Sterols are group of steroids with a multiple-ring structure and hydroxyl group at the third position of the A ring. They naturally occur in live organisms membranes and are important class of organic molecules. The most abundant sterol in mushrooms is ergosterol ($(3\beta,22E)$ -Ergosta-5,7,22-trien-3-ol; *Fig. 1.11*) contain 28 carbon atoms (Teichmann et al., 2007), differing from major plant phytosterol in having two double bonds in the sterol ring structure instead of one. Various minor sterols, present in fungi, have been isolated, including fungisterol ($(3\beta, 5\alpha, 22E)$ -ergosta-6, 8, 22-trien-3-ol; *Fig. 1.11*), ergosta-5, 7-dienol, 24-methyl cholesterol and methylene cholesterol. The fungal sterols provide characteristic functions that are necessary for vegetative growth. (Mattila et al., 2002).

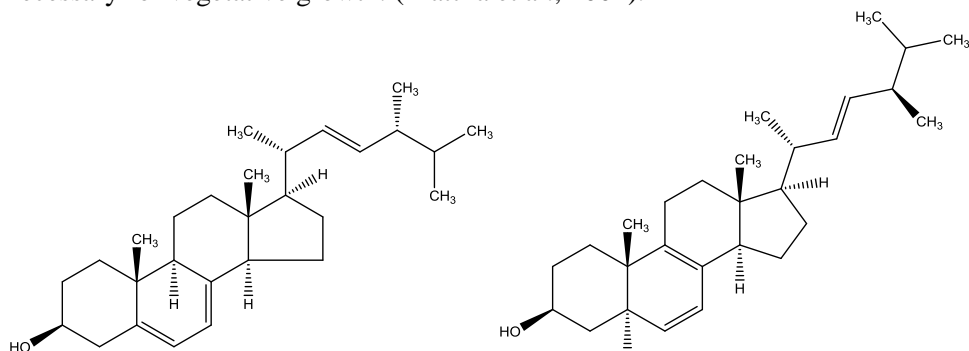


Fig. 1.11 Structure of ergosterol, fungisterol

Other known sterol represented in mushrooms is ergocalciferol or Vitamin D₂. This essential compound is the synthetic form of vitamin D₂ that can be formed from the mushroom sterol, ergosterol by UV irradiation and it is assumed to have the same biological activity as cholecalciferol (vitamin D₃). It plays a vital role in calcium metabolism and bone mineralization especially for children (Jasinghe and Perera, 2005)

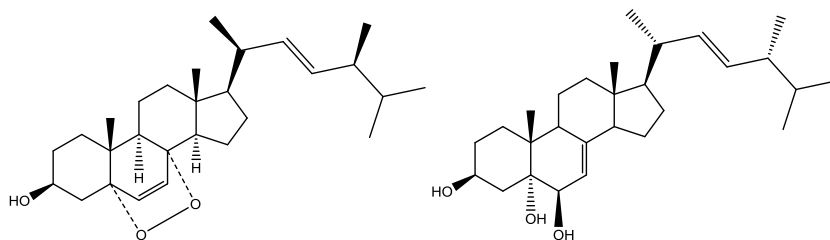


Fig. 1.12 Structure of 5 α ,8 α -epidioxy (22E,24R)-ergosta-6,22-dien-3 β -ol, cerevisterol

In the course of searching for new bioactive products with developed properties the sterol (5 α ,8 α -epidioxy-(22E,24R)-ergosta-6,22-dien-3 β -ol; Fig. 1.12) isolated from *Paxillus panuoides* (Gao et al, 2002) and *Agrocybe aegerita* (Zhang, Mills and Nair, 2003) possessed potent anticancer and antioxidant activity. The ectomycorrhizal *Tuber* species, and related fungi also some *Volvariella* species contain cerevisterol (22E,24R)-ergosta-7,22-dien-3 β ,5 α ,6 β -triol; Fig. 1.12), which have cytotoxic activity against malaria (Li et al., 2007, Jinming, Lin and Jikai, 2001, Mallavadhani et al., 2006).

1.2.1.4. Terpene compounds in mushrooms

Terpenes are constituted from multiple isoprene units, which has the molecular formula (C₅H₈)_n. Terpenes are known as an important diversity of naturally occurring bioactive molecules produced by many higher fungi. Particularly diterpenoid, triterpenoid, and sesquiterpenoid are the typical representatives of terpenes with interesting biological activities of mushrooms.

Ganoderma lucidum, well-known traditional Chinese medicine in eastern Asia, is used as a folk panacea for the treatment of cancer, hepatitis, chronic bronchitis, asthma, hemorrhoids, and fatigue symptoms, and has contributed over 130 highly oxygenated and pharmacological active lanostane-type triterpenoids from its fruiting bodies, mycelia, and spores, many of which exhibited cytotoxic activity against various tumour cell lines (Paterson, 2006). Three triterpenes lanostane-type aldehydes lucialdehydes B,C ((24E)-3,7-dioxo-5 α -lanosta-8,24-dien-26-al; (24E)-3 β -hydroxy-7-oxo-5 α -lanosta-8,24-dien-26-al; Fig. 1.13) and lucidenic acid N ((3 β ,5 α ,7 β)-3,7-dihydroxy-4,4,14-trimethyl-11,15-dioxochol-8-en-24-oic acid; Fig. 1.14),

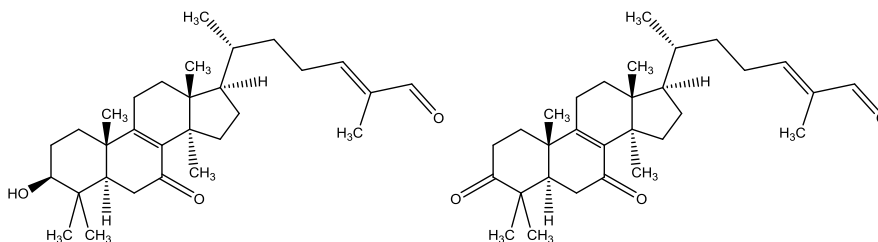


Fig.1.13 Structure of lucialdehydes B,C

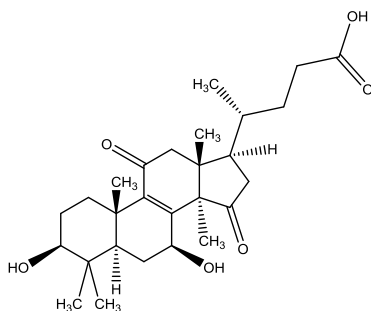


Fig 1.14 Structure of lucidenic acid N

were successively isolated from *G. lucidum* and showed significant cytotoxic effects against cancer cells (Wu et al., 2001, Gao et al., 2002). Therefore, the mushroom *G. lucidum* is considered as a rich mine of triterpenoids bioresource.

Most members of the genus *Lactarius* abundantly contain a milky juice, which appears when the fruiting bodies are injured. Sesquiterpenes represent an important biological role in the great majority of *Lactarius* species, as essential for the pungency and bitterness of the milky juice and the change in color of the latex on exposure to air and constituting a chemical defense system against various invaders such as bacteria, fungi, animals, and insects. In the process of bioactive metabolites investigation for *Lactarius* species sesquiterpene, named as rufuslactone was isolated, from the mushroom *L. rufus*. Rufuslactone ((9S,13R)-8,11,11-Trimethyl-4,14-dioxatetracyclo[6.5.1.0^{2,6}.0^{9,13}]tetradec-2(6)-en-5-one; Fig. 1.15) is an isomer of a previously described lactarane (3,8-oxa-13-hydroxylactar-6-en-5-oic acid γ -lactone) from *L. necatar* and possessed potent antifungal activity (Luo et al., 2005). Subvellerolactones B and D (Fig. 1.15) structurally unusual lactarane sesquiterpenoids, were isolated from the fruiting bodies of *Lactarius subvellerus*. Subvellerolactones B exhibited cytotoxicity against the lung carcinoma, skin melanoma and human lung cancer and subvellerolactones B, E showed cytotoxicity against skin melanoma and human tumour cells (Kim et al., 2010).

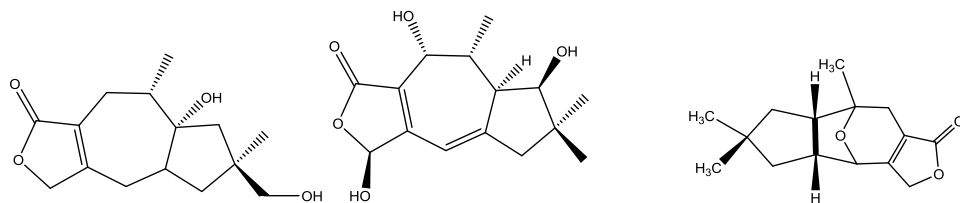


Fig. 1.15 Structure of subvellerolactones B,D and rufuslactone

1.2.1.5. Miscellaneous compounds in mushrooms

Naturally occurring small diversified molecules are recognized as an important source of bioactive substances. Miscellaneous group have wide range compounds including phenolics, acids, polyphenols and others. Paxillaceae family is typical representatives possessing the rich secondary metabolites *p*-terphenyls. Approximately 20 *p*-terphenyls, named as curtisians A–Q (Fig. 1.16), were successively isolated from *P. curtissii* growing widely in East Asia and South Europe on decayed pine trees. Most of the *p*-terphenyl compounds exhibited attractive antioxidant activities against lipid peroxidation or radical-scavenging activity against DPPH (Quang et al., 2003a; Quang et al., 2003b).

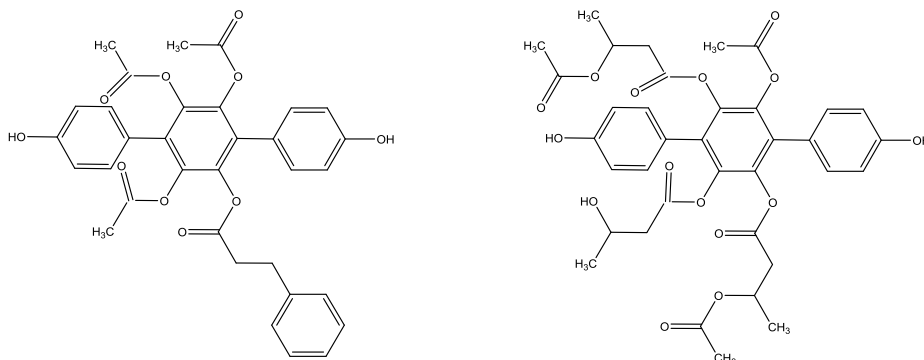


Fig. 1.16 Structure of curtisian B and C

Ellagic acid and its derivatives are widely distributed in plants, but are rare in fungi. The basidiomycete *Russula nigricans* whose fruiting bodies have been found to show antitumor activity was identified as a phenolic compound based on the ellagic acid skeleton. Nigricanin (3,8-dihydroxy-10-methoxy[1]benzopyrano[5,4,3-cde][1]benzopyran-5(10H)-one; Fig. 1.17) considered as the first ellagic acid like compound found in higher fungi.

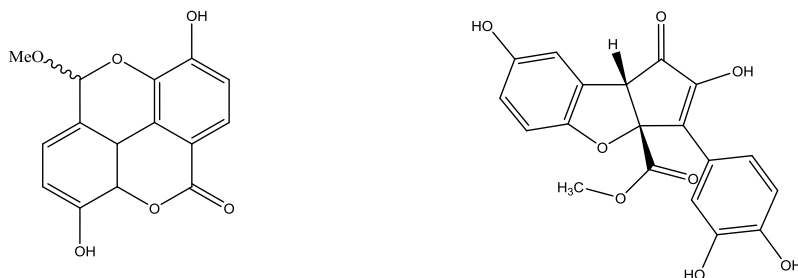


Fig. 1.17 Structure of nigricanin and suillusin

Other unique benzofuran named suillusin was isolated from the fruiting body of the mushroom *Suillus granulatus*. Suillusin (1*H*-cyclopenta[*b*]benzofuran; Fig. 1.16) which is biogenerated from polyporic acid indicate potential antioxidant activity and specific cytotoxic effect against human cancer also melanoma (Yun et al., 2001).

1.3. BRIEF REVIEW OF SELECTED MUSHROOMS SPECIES AND THEIR SECONDARY METABOLITES

This research focuses on wild growing mushroom species from Midi-Pyrénées, France (Table 2.1). Literature surveys on the selected coastal and forest mushrooms species with potential activity or biological compounds are rather scarce or not described at all.

Agaricus are well known mushroom genus with possibility over 200 species all over the world (Kerrigan, Challen and Burton, 2013). *Agaricus pseudoprattensis* possess unpleasant odor provided by phenolic compounds composition as well as fatty acids and their esters (Petrova et al., 2007). Other species, *Agaricus devoniensis* was extracted with increased polarity of solvents obtain microbial and antioxidant activity (Al-Fatimi et al., 2005). Phenolic and almond odor *Agaricus fissuratus* have shown potential antitumor activity (Didukh and Mahajna, 2005).

Boletus is a genus of mushrooms, consisting over 100 species. All of the forest species gathered worldwide, the edible mushrooms from the *Boletus* genus are the most popular and frequently harvested in European countries, due to their particular aroma, taste and texture (Heleno et al., 2011). It was found the chemical, antioxidant and nutritional values of *Boletus impolitus* collected in the Northeast Portugal (Pereira et al., 2012). Also antimicrobial potential of methanol fraction extract (Nikolovska-Nedelkoska et al., 2013) and monoterpenic volatile compounds of fresh *Boletus lupines* were determined (Breheret et al., 1997). Several carotenoids were identified in edible species of *Boletus luridus* (Czeczuga, 1978); however studies on the antioxidant properties of selected *Boletus* species are scanty.

Gilled genus *Tricholoma* are generally growing in woodlands or sandy dunes. *Tricholoma caligatum* forms a small and fuscous to blackish fruit body and grows in the Mediterranean region (Murata et al., 2013). Various aromatic derivatives were identified in *T. caligatum* (Fons et al., 2006). Pine forest or sea-side sand dunes mushroom *Tricholoma auratum* have active anti-osteoporosis ergosterol derivative (Hata et al., 2002) and have microbial activity (Yamaç and Bilgili, 2006). *Tricholoma columbetta* is edible and can be consumed fresh, dry or pickled. A cyclopentene derivative columbetdione (Vadalà et al., 2003) and endopeptidase (Lamaison, Pourrat and Pourrat, 1980) were found in its fruiting bodies, while ethyl acetate extracts of *T. columbetta* were shown to possess nematocidal activity against *Caenorhabditis elegans* and antibacterial activity against *Bacillus brevis* (Stadler and Sterner, 1998). Unfortunately antioxidant activity of bioactive components on selected species not detected.

Clitocybe odora known as anise odor mushroom rich in ρ -anisaldehyde, benzaldehyde and other volatile compounds (Malheiro et al., 2013) have valued

chemical composition, besides exhibited potential antioxidant and antimicrobial properties (Vaz et al., 2011, Suay et al., 2000).

The most species-rich mushroom genus known today is *Cortinarius*. Some species of *Cortinarius* possess chemical compounds that have significant biological properties, such as alkaloids activity against Alzheimer's disease of species *Cortinarius infractus* (Bronz et al., 2007, Geissler et al., 2010, Stefani, Jones and May, 2014).

Phaeolus schweinitzii (Fr.) is a common root and butt pathogen of conifers in North America and Eurasia producing a strong, water-soluble pigments possessing five intensive colours: olive-brown, olive-grey, dark-brown, brownish-grey and linoleum-brown (Cedano, Villaseñor and Guzmán-Dávalos, 2001). This mushroom also known as *Polyporus schweinitzii* has a wide host range infecting species of Cupressaceae (*Thuja*), Pinaceae (*Abies*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga*, *Tsuga*) and Taxaceae (*Taxus*) in natural forests. Sessile mushroom has a velvety, brown to olive spongy cap when mature, a yellowish brown or orange with dark brown pore surface and margin when young. The pores are angular and fairly large, and the fairly thin, flexible flesh is brown to reddish brown. Small stipe is solid and fibrous with tomentose surface (Simpson and May, 2002). In medicine *P. schweinitzii* has been recorded to possess bioactivity against tumour cells *in vivo* and showed antimicrobial activity. Only one active metabolite-hispidine was isolated from acetone extract of *P. schweinitzii* (Ueno et al., 1964). There were no reports describing secondary metabolites and their activity of *P. schweinitzii*. During our ongoing efforts to discover active substances researchers in China described potent radical scavenging capacity of five new hispidine derivates phaeolschidin A–E together with the known compounds pinillidine and hispidin from ethanol extract (Cedano et al., 2001, Han et al., 2013).

Inonotus hispidus is a parasitic fungus preferably living on deciduous trees such as *Fraxinus*, *Quercus*, *Sorbus* and *Malus*. It has been used as a traditional medicine for treating dyspepsia, cancer, diabetes and stomach problems in the northeast region and Xinjiang province of China (Awadh Ali et al., 2003). *I. hispidus* contains polyphenol pigments with styrylpyrone skeleton, which were reported to exhibit antimicrobial, antioxidant, antiviral and anti-inflammatory activities. Two natural antioxidants, named inonotusin and hispidin were isolated from the methanolic extract of the fruit bodies and showed antioxidant and cytotoxic activity against human breast carcinoma cells (Zan et al., 2011).

Xerocomus chrysenteron is an edible mushroom occasionally harvested in autumn. The lectin was identified in *X. chrysenteron* (Birck et al., 2004), while its methanolic extract was reported to possess antioxidant activity (Sarikurkcu, Tepe and Yamac, 2008, Heleno et al., 2012).

Hydnellum ferrugineum has red spore deposit and is easily recognized. *Hydnellum* spp. are regarded as “nitrogen sensitive” organisms (Van der Linde, Alexander & Anderson, 2008; (Ainsworth et al., 2010, van der Linde, Alexander and Anderson, 2008) and have become a concern of European conservation.

Biological active compounds interact differently with microorganisms indicating that different components may have different modes of action or that the metabolism of some microorganisms are better able to overcome the effect of the compound or adapt to it. Although it is important to mention that single compound may not be responsible for the observed activity but rather of compounds interacting in an additive or synergistic manner (Paiva et al., 2010).

Considering that there are about 7000 poorly studied until now wild mushrooms species, it may be concluded that mushrooms have a good potential in searching valuable bioactive substances for developing natural preparations for multipurpose goals. For increasing practical exploration of mushroom diversity in various regions they should be more comprehensively and systematically studied, e.g. by using various activity guided assays. Regardless the above-cited articles, the reports on the properties and the presence of bioactive compounds in the selected in this study mushroom species are rather scarce. Therefore, the primary aim of the present work was to evaluate antioxidant, antimicrobial cytotoxic properties and biological active compounds of the 32 underinvestigated wild mushroom species by applying a more systematic approach in order to obtain scientific information, which is required for their preliminary valorization as a source for human nutrition, production of functional ingredients and medicinal applications as biologically active substances.

2.3.4. THE POTENTIAL ACTIVITY OF MUSHROOM SUBSTANCES AND THEIR RECOVERY

Historical traditions and the extensive research prove the preventive and therapeutic properties of many mushroom species. The natural compounds of mushrooms are potentially important not only in medicine, with specific health effects and cure of diseases associated with oxidative damage. But it is also important for the food industry because they obstruct oxidative degradation of lipids and thereby improve the quality and nutritive value of food products, including dietary supplements and products sometimes referred as functional foods. Therefore, search for effective and non-toxic natural antioxidants and other bioactive molecules have become a regularly increasing topic. Synthetic antioxidants such as butylated hydroxyanisole, tert-butylated hydroxyquinone and butylated hydroxytoluene are radical scavengers but are usually associated with negative side effects (Woldegiorgis et al., 2014). Affect the free radical damage by naturally occurring antioxidants from several sources either plant or fungi kingdom is becoming one of the most appreciate models of modern therapy. Antioxidants with the source of healthy compounds are present in all biological systems and mushrooms are not exception. In literature antioxidatively active components can be ranked as primary or long-term antioxidants and as secondary or processing antioxidants. Primary antioxidants contain hindered phenols and secondary aryl amines and secondary antioxidants contain organophosphites and thioesters; thus the primary antioxidants are active radical scavengers or hydrogen donors or chain reaction breakers while the secondary ones are peroxide degeneraters (André et al., 2010).

Since the antioxidant capacity of complex biological extracts is usually determined by a mixture of various antioxidatively active constituents, which may act by different mechanisms and sometimes possess synergistic effects, the reliability of the evaluation of overall antioxidant potential of any plant material increases by applying several assays (Frankel and Meyer, 2000, Laguerre, Lecomte and Villeneuve, 2007). ABTS^{•+} and DPPH[•] scavenging, FRAP, ORAC and TPC assays are the most common methods for determining *in vitro* antioxidant capacity of plant origin substances. ORAC, TPC measured with Folin-Ciocalteu reagent and one of the single electron/hydrogen atom transfer assays (SET or HAT) should be recommended for the representative evaluation of antioxidant properties (Huang, Ou and Prior, 2005). DPPH[•] scavenging method is mainly attributed to the SET assays; however, quenching of DPPH[•] to form DPPH-H is also possible. Other SET based methods include ABTS^{•+} decolourisation assay and ferric ion reducing antioxidant power (FRAP) assay and the TPC assessment using Folin-Ciocalteu reagent. ORAC assay evaluates radical chain breaking antioxidant activity via HAT and measures antioxidant inhibition induced by peroxy radical oxidation.

Frequently, solvent extraction techniques are used for bioactive compounds recovery. Depending on extracted compound or class of compounds important factors in the process are solvent type, extraction time and temperature. The polarity of solvent should be the similar class as desirable bioactive molecule. Efficient extraction of antioxidants, antimicrobials and other biologically active molecules requires the use of solvents with different polarities: hydrophilic compounds are better soluble in polar solvents such as methanol, water, whereas cyclohexane or dichloromethane are preferable for isolating lipophilic molecules. Two main techniques may be applied for exhausting isolation of various biological active compounds from material, namely parallel extraction of initial material with different solvents or sequential fractionation with increasing polarity and dielectric constant solvents.

1.4.1. Antioxidants in mushroom and their mode of action

Free radicals are produced in the normal natural metabolism of aerobic cells as oxygen reactive species (ROS). The essential conditions for normal organism functioning and maintains of equilibrium free radicals are neutralized by cellular antioxidant defences (enzymes and non-enzymatic molecules). However, the overproduction of ROS or the loss of the cell antioxidant defences is known as oxidative stress and in this case, the excess ROS may oxidize and damage cellular lipids, proteins and DNA, leading to their modification and inhibiting their normal function. This disequilibrium of radicals has been related to more than one hundred diseases including several kinds of cancer, diabetes, cirrhoses, cardiovascular diseases, neurological disorders, aging process and others (Ferreira et al., 2009). Free radicals derived from molecular Oxygen (O₂) are usually known by reactive Oxygen species (ROS) and generally described as unstable most important class radical species generated in living systems. Superoxide anion (O₂^{•-}) is the “primary” ROS radical and mostly produced in mitochondria, due to a small but continuous

“leak” of the electrons in the mitochondrial electron transport system (ETS). This anion can also be formed by different endogenous enzymatic systems present in the cell like NADPH oxidases and xanthine oxidase. However $O_2^{\bullet -}$ possibly interact with other bioactive molecules and are generating “secondary” ROS, such as hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^{\bullet}). Hydroxyl radical is described to be the most toxic among all radicals and being responsible for the affect to DNA molecules, although it has a very short existence period (Halliwell and Gutteridge, 1990). Hydroxyl radical is the neutral form of the hydroxide ion and it is formed by an electron transfer from transition metals to H_2O_2 , and interacts with biomolecules enzymes immediately after generation (Valko et al., 2007). Free ROS radicals are susceptible to attack membrane lipids, where lipid peroxidation usually begins with the extraction of a hydrogen atom from a polyunsaturated lipid (LH) chain, through the action of reactive species such as HO^{\bullet} . This generates a highly reactive lipid radical (L^{\bullet}) that can interact with oxygen to form a peroxy radical (LOO^{\bullet}), which not influenced with antioxidants, will react with other neighbouring lipids producing hydroperoxides lipids (LOOH) and can easily be decomposed to form new L^{\bullet} radicals, initiating a process that is known as chain propagation reactions (McCord, 2000, Abreu, Santos and Moreno, 2000).

Other important radicals are reactive Nitrogen species (RNS), were nitric oxide (NO^{\bullet}) is generated in biological tissues by specific nitric oxide synthases (NOS), which metabolise arginine to citrulline. Nitric oxide is an important oxidative biological molecule with a different type of physiological processes, including neurotransmission, blood pressure regulation, defence mechanisms, and regulation of immune response. The RNS abundance is called nitrosative stress and may lead to nitrosylation of proteins and affect their normal function. Nitric oxide jointly with superoxide anion oxidation in cell immune system may influence inflammatory processes. Whereas NO^{\bullet} can react with O_2 and produce a potent oxidising agent peroxy nitrite anion ($ONOO^{\bullet}$), which can cause DNA fragmentation and lipid oxidation (Ferreira et al., 2009, Carr, McCall and Frei, 2000).

Exposure to free radicals from a variety of sources has led organisms to the development of a series of different endogenous enzymatic antioxidant defences in the organism which include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx), glutathione reductase (Gred), glutathione (GSH), α -tocopherol (vitamin E), ascorbic acid (vitamin C), lipoic acid and other antioxidants (Valko et al., 2007, Fang, Yang and Wu, 2002). Besides endogenous defences, antioxidant supplements or antioxidant-containing foods may be used to help the organism to reduce oxidative damage or to protect food quality by preventing oxidative stress. A multitude of natural antioxidants have already been isolated from different kinds of biological materials and mushroom bioactive substances are identified as one of possible potentials. The benefit of using mushrooms over plants as sources of bioactive compounds are that often the fruiting body can be produced in short time, the mycelium may also be rapidly produced in liquid culture and the culture medium can be manipulated to produce optimal quantities of biologically active substances (Ferreira et al., 2009).

The antioxidants found in mushrooms are mainly represents as phenolic compounds (phenolic acids and flavonoids), followed by tocopherols, ascorbic acid and carotenoids. Phenolic compounds are secondary metabolites, constitute from aromatic hydroxylated compounds, possessing one or more aromatic rings with one or more hydroxyl groups, usually found in vegetables, fruits and many food sources. Natural bioactive molecules are accumulated from biological sources as end-products and can range from relatively simple molecules (phenolic acids, phenylpropanoids, flavonoids) to highly polymerised compounds (lignins, melanins, tannins), such as flavonoids and phenolic acids representing the most common group in mushrooms. Phenolic acids can be divided into two groups (*Fig. 1.18*): one of hydroxybenzoic acids derived from non-phenolic molecules benzoic acid, usually occurred in the bound form and are typical component of a complex structure like lignins and hydrolyzable tannins, also linked with sugars or organic acids in plants.

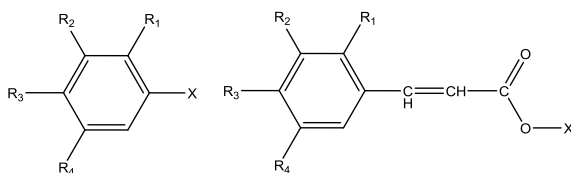
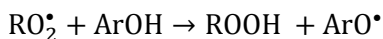


Fig. 1.18 Benzoic and cinnamic acid derivatives

Other group is hydroxycinnamic acids derivative of cinnamic acid a commonly present in the bound form, connected to cell-wall structure components, such as cellulose, lignin and proteins, also to tartaric or quinonic acids (Liu, 2004; Ferreira et al., 2009).

There are important factors which determine the antioxidant effectiveness and potential of natural phenolic compounds: stability of natural antioxidant formed during radical reaction, involvement of the phenolic hydrogen in radical reaction and chemical substitutions present on the structure. The substitutions on the structure are one of most important aspects with ability of active phenolic molecule to participate in the control of radical reactions and form resonance-stabilized antioxidants radicals from natural origin as follows:



Antioxidant activity of phenolic acids depends on phenolic hydrogens, hydroxyl substitutions at *ortho* and *para* positions. The stability of phenoxy radical increases if intermolecular hydrogen bonds are formed by *ortho* position, also the second hydroxyl group possess stronger antioxidant activity, than compounds containing a methoxy (OCH_3) group (Ferreira et al., 2009).

Flavonoids represent a large group of phenolic compounds (*Fig.1.19*), which consist of two aromatic rings (A and B rings) linked by a three carbon chain that is usually in an oxygenated heterocycle ring (C). These compounds naturally occur in

plants, fruits, vegetables, grains, barks, roots and have been linked to reduce the risk of major chronic diseases (Liu, 2004).

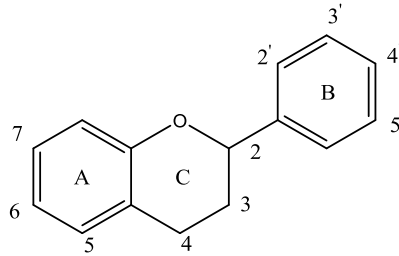


Fig.1.19 The generic structure of flavonoids.

Several classes of flavonoids identified in wild mushrooms are described on the basis of differences in the generic structure of the heterocycle C ring and can be classified as flavonols, flavones, flavan-3-ols, flavanones and isoflavones (Fig. 1.20) (Ferreira et al., 2009).

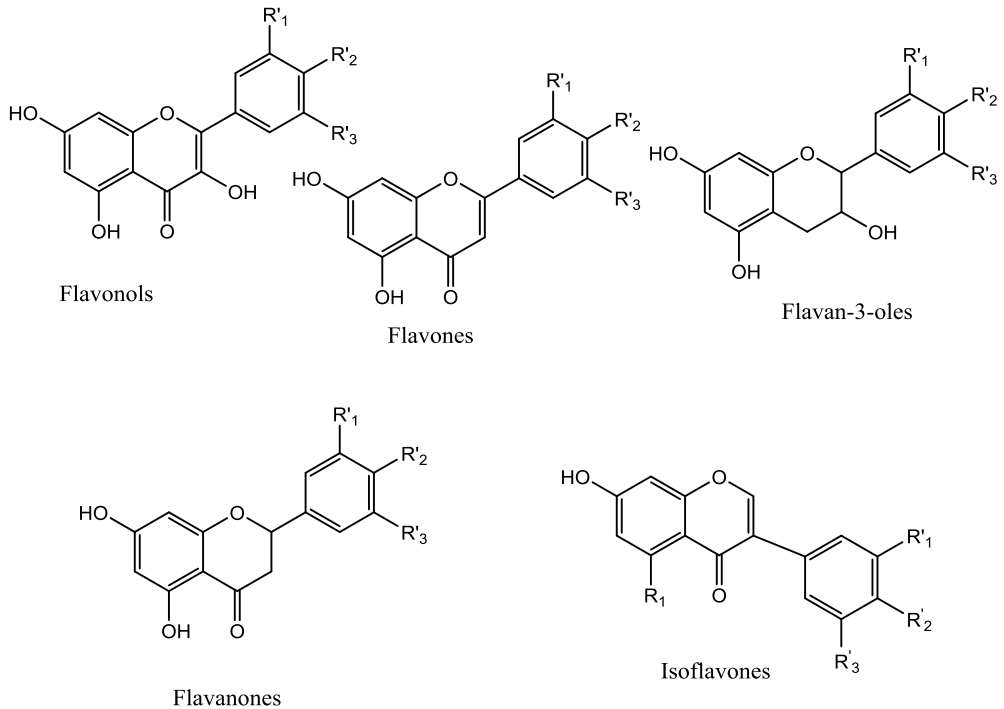


Fig.1.20 Flavonoids classes identified in wild mushrooms

Flavonoids are characterized as health-promoting molecule, including antioxidant anti-inflammatory and anti-proliferative activities, with in inhibition

mechanisms of bioactivating enzymes, or induction of detoxifying enzymes (Le Marchand, 2002). Protective effect against cardiovascular diseases was the first mechanism of action studied of flavonoids as antioxidants. Flavonoids multifunctional properties have been shown to be highly effective radical scavengers mostly of oxidizing molecules, including singlet oxygen and various free radicals, which are possibly responsible for DNA damage and tumour promotion (Wright, Johnson and DiLabio, 2001). Phenolic compounds from wild mushrooms represents as one of the most effective antioxidants with reduced risk of chronic diseases due to their ability to reduce agents by donating hydrogen and quenching singlet oxygen. Antioxidant properties of tocopherols (α -, β -, δ -, γ -tocopherols) found in mushrooms also play a vital role in cancer and cardiovascular diseases.

Vitamin E is one of the tocopherol form which reacts with peroxy radicals produced from polyunsaturated fatty acids in membrane phospholipids or lipoproteins to yield a stable lipid hydroperoxide. Whereas, they act as antioxidants by donating a hydrogen atom to peroxy radicals of unsaturated lipid compound, forming a hydroperoxide and a tocopheroxyl radical. This active radical reacts with other peroxy or tocopheroxyl radicals forming better stability of foods, as well as in the antioxidative defence mechanisms of biological systems (Lampi et al., 1999)

Ascorbic acid, also known as vitamin C associate with various oxidative stress related health problems such as heart disease, stroke, cancer, several neurodegenerative diseases and cataractogenesis. Vitamin C is an effective against superoxide, hydroxyl radical, hydrogen peroxide, peroxy radical and singlet oxygen as well as can protect biomembranes against lipid peroxidation damage. This antioxidant eliminates peroxy radicals in the aqueous phase before the latter can initiate lipid peroxidation. Vitamin C and vitamin E interact synergistically at the membranecytosol interface to regenerate membrane-bound oxidized vitamin E (Sies, Stahl and Sundquist, 1992).

Overall phenolic compounds, tocopherols, ascorbic acid defined as antioxidatively active compounds which could be found in wild mushrooms and used as functional ingredients or health promoters, namely against chronic diseases related to oxidative stress.

1.4.2. Antimicrobials in mushroom and their mode of action

Mushrooms are forced to accumulate antibacterial and antifungal compounds in their fruiting body to survive in natural environment. It is not surprising that antimicrobial compounds with potential activities isolated from many mushrooms species could be a benefit for humans, as rich source of natural antibiotics with ability combat bacteria and viruses (Lindequist et al., 2005). During antimicrobial mechanism of action biological active molecules penetrate and interfere with cell membrane lipids, and affect normal barrier functions. This may cause membrane fusion and leakage of intermembranous material aggregation, which may damage and disarray normal functions, determining cell death (Ikigai et al., 1993, Sato et al., 1996). Bioactive molecules terpenes are described as membrane disruption promoters, coumarins cause reduction in cell respiration and tannins act on

microorganism membranes as well as bind to polysaccharides or play as cell enzymes promoting inactivators (Paiva et al., 2010). Less hydrophylic molecules, for instance flavonoids lacking the hydroxyl groups on their B ring are more active inhibitors against microorganisms than those molecules with – OH groups. (Chabot et al., 1992). Either methyl group number on C ring of the flavonoids increases antimicrobial activity (Ibewuiké et al., 1997). The aliphatic side on the ring A defined as more polar flavones with enlarged potential antibacterial activity (Cowan, 1999). However antimicrobial activity of compounds vary dependant on microorganism strain and membrane structure.

Several biologically active molecules (polyketides, terpenes, steroids and phenolic compounds) extracted from mushroom revealed antifungal and antibacterial activity, namely against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*. Sesquiterpenoid hydroquinones isolated from well know *Ganoderma* species *Ganoderma pfeifferi* inhibited the growth of methicillin-resistant *S.aureus*, either the same mushroom extract prevent the growth of microorganisms responsible for skin problems(Mothana et al., 2000). Applanoxidic acid A, belongs to triterpens class bioactive molecules were isolated from *Ganoderma annulare*, possessed potential antifungal activity against *Trichophyton mentagrophytes*. Several steroids (5 α -ergosta- 7, 22-dien-3 β -ol or 5,8-epidioxy-5 α ,8 α -ergosta-6, 22-dien-3 β -ol) isolated from *Ganoderma applanatum*, proved to be moderately active against number of Gram positive and Gram negative microorganisms (Smania et al., 1999). Oxalic acid has inhibitory effect for microbials isolated from *Lentinula edodes* mushroom against *S. aureus* and other bacteria (Lindequist et al., 2005).

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Research objects

Twenty four coastal-dune mushroom species were collected at mature form in South-western region of France in autumn 2011 (*Table 2.1*; *Table 2.2*). Six forest mushroom species (*Phaeolus schweinitzii*, *Inonotus hispidus*, *Tricholoma columbetta*, *Tricholoma caligatum*, *Xerocomus chrysenteron*, *Hydnellum ferrugineum*) were harvested in Midi-Pyrénées region of France in autumn 2009. Additionally matured *Phaeolus schweinitzii* mushroom was collected in 2011 and 2012. Taxonomic identification was carried out by the Research Unit “Mycology and Food Safety” at INRA Research Centre of Bordeaux-Aquitaine and Mycologist Association of Faculty of Pharmacy of Toulouse University. All freeze-dried (Lyophilisateur pilote LPCCPLS15, Cryotec, Saint-Gély-du-Fesc, France) mushrooms were stored in paper bags in a desiccator at ambient temperature (<30 °C) for further analysis. All experiments were performed in one year duration.

Table 2.1 List of studied mushroom families

Family	Number of species	
	Forest species	Coastal-dune species
Agaricaceae	9	
Tricholomataceae	4	2
Boletaceae	4	1
Russulaceae	2	
Cortinariaceae	2	
Polyporaceae		2
Amanitaceae	1	
Gyroporaceae	1	
Bankeraceae		1
Strophariaceae	1	

2.1.2. Reagents

Stable 2,2-*di*-phenyl-1-picrylhydrazyl hydrate radical (DPPH[•], 95%), gallic acid, anhydrous sodium carbonate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 97%), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), fluorescein (FL), [2,2'-azobis(2-amidino propane) dihydrochloride (AAPH)], cyclohexane, dichloromethane acetonitrile, ethanol, methanol, vanillin, 2-aminoethyl diphenylborinate, polyethylene glycole, macragol 4000, Sephadex LH-20, amphotericin B, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), isopropanol, sodium dodecyl sulphate, methanol, chloroform, TLC plates (silica gel G60 F₂₅₄), medium (RPMI 1640) mouse macrophage cell line (J774A.1 cell line) were from

Sigma-Aldrich (Steinheim, Germany); 2.0 M Folin–Ciocalteu phenol reagent, KCl, NaCl, Na₂HPO₄, Na₂CO₃ and K₂S₂O₈, PBS (0.1 M phosphate containing 0.05 M NaCl, pH-7.2) were from Merck (Darmstadt, Germany); KH₂PO₄ from Jansen Chimica (Beerse, Belgium); 98% acetic acid from Lachema (Brno, Czech Republic) and agricultural origin ethanol (96.6 %) from Stumbras (Kaunas, Lithuania); 2,4,6-tripyridyl-s-triazine (TPTZ) was from Fluka Chemicals (Steinheim, Switzerland). Sulfuric acid, bismuth nitrate, glacial acetic acid, potassium iodide were from Jansen Chimica (Beerse, Belgium); Nutrient agar (Bit Phar. acc EN 12780:2002) was from Scharlau (Barcelona, Spain). *Bacillus cereus* (330) was from Microbial Strain Collection of Latvia (MSCL); *Pseudomonas aeruginosa* (27853) *Staphylococcus aureus* (25923), *Escherichia coli* (25922) were from American Type culture Collection (ATCC). *Leishmania infantum* MHOM/MA/67/ITMAP-263 (CNR *Leishmania*, Montpellier, France). Milton sterilizing fluid (2%, w/v and the 16.5% w/v sodium chloride solution) was from Louzy, France. Steady Glow reagent was from Promega (California, USA). *Leishmania infantum* MHOM/MA/67/ITMAP-263 (CNR *Leishmania*, Montpellier, France). *Haemonchus contortus* (stored 4°C for 2 months before use) and *Trichostrongylus colubriformis* (stored 4°C for 1,5 before use) the L₃ (third stage larvae) were obtained from a donor sheep with a monospecific infection (INRA, France).

Table 2.2 List of studied mushroom species

Botanical name	Ecology	Family	Characterization	Habitat	Edibility	References
<i>Agaricus menieri</i>	Saprotrophic	Agaricaceae	Strong phenolic odour, cap chalk whited, yellow discolouring by handling or cutting; belongs section <i>Xanthodermatei</i> ;	Internal side of high dunes, close to plants of <i>Ammophiletum spp.</i> ;	Inedible	(Lacheva, 2013) (Kerrigan et al., 2005)
<i>Agaricus pseudopratisensis</i>	Saprotrophic	Agaricaceae	Strong phenolic odour, brown cap squamous and frequently rufous flesh; belongs section <i>Xanthodermatei</i> ;	Lawned rear dunes, anthropic or grassy areas;	Inedible	(Petrova et al., 2007, Callac and Guinberteau, 2005)
<i>Agaricus freirei</i>	Saprotrophic	Agaricaceae	Strong phenolic odour, faint yellowish discolouring flesh of the stipe quickly followed by a red vinaceous discolouring; cap colour varying from mahogany brown to chocolate brown; section <i>Xanthodermatei</i> ;	Wooden dunes, sandy calcareous soil either in the <i>Pino-Quercetum ilicis</i> or under <i>Cupressus macrocarpa</i> ;	Inedible	(Kerrigan et al., 2005)
<i>Agaricus devoniensis</i>	Saprotrophic	Agaricaceae	Colour whitish with irregular brownish areas; odour mushroomy; unchanging when cut, latter becoming brownish; section <i>Bivelares (Duploannulati)</i> ;	Fixed dunes close to <i>Phleo Tortuletum</i> , in fairly level areas of dark dunes;	Edible	(Kerrigan, Callac and Parra, 2008)
<i>Agaricus fissuratus</i>	Saprotrophic	Agaricaceae	Phenolic and almond odour; Cap white and smooth hemispherical shape;	Wooden dunes close to <i>Pino-Quercetum ilicis</i> and thickets of	Edible	(Guinberteau)

Botanical name	Ecology	Family	Characterization	Habitat	Edibility	References
<i>Agaricus coniferarum</i>	Saprotrophic	Agaricaceae	Cap blushing;	dunes; Thrives on humus;		
<i>Leucoagaricus litoralis</i>	Saprotrophic	Agaricaceae	Cap first convex, latter spread out; pink colour with bleaching shade;	Semi fixed dunes at sagebrush carpet or <i>Cupressus macrocarpa</i> ;	Inedible	(Guinberteau)
<i>Leucoagaricus subolivaceus</i>	Saprotrophic	Agaricaceae	Cap green bronze with bleaching shade; sensitive for sunlight becomes lighten;	Semi fixed dunes at sagebrush carpet or <i>Cupressus macrocarpa</i> ;	Inedible	(Guinberteau)
<i>Sericeomyces subvolvatus</i>	Saprotrophic	Agaricaceae	Cap silky smooth, whitish and creamy in the middle;	Semi fixed dunes, grassy areas or degraded dunes;	Inedible	(Guinberteau)
<i>Gyroporus ammophilus</i>	Mycorrhizal	Gyroporaceae	Cap often shapeless, wavy and velvety surface; similar to blond boletus;	Beside wooden dunes close to <i>Cistus salvifolius</i> or <i>Pino-Quercetum ilicis</i> ;	Inedible	(Guinberteau)
<i>Leccinum (Boletus) lepidus</i>	Mycorrhizal	Boletacea	Cap broad, reddish –brown; stem white and becomes darker in the base;	Wooden dunes or close to <i>Pino-Quercetum ilicis</i> ;	Edible	(De Dominicis and Barluzzi, 1983)

Botanical name	Ecology	Family	Characterization	Habitat	Edibility	References
<i>Boletus impolitus</i>	Mycorrhizal	Boletacea	Robust stature and floccose stem; smell of iodine at the stipe base when cut; truncated stipe becomes yellow;	Wooden dunes or close to <i>Pino-Quercetum ilicis</i> ;	Inedible	(Hills, 2008)
<i>Boletus luridus</i>	Mycorrhizal	Boletacea	Cap convex velvety or nearly bald; Stem yellowish above and reddish below;	Wooden dunes, sandy calcareous soil either in the <i>Pino-Quercetum ilicis</i> ;	Edible	(LI, WU and XU, 2009)
<i>Boletus lupinus</i>	Mycorrhizal	Boletacea	Cap dry, smooth, pale grey to very pink pale; stem bright yellow, discolouring; tinted orange red or reddish in places;	Warm broadleaf dune forests close to oaks <i>Quercus</i> or sweet chestnut <i>Castanea</i> ;	Inedible	(Guinberteau)
<i>Xerocomus chrysenteron</i>	Mycorrhizal	Boletacea	Cap velvety brown to cracked brown or olive brown; stem yellow to reddish	Hardwoods; especially oak; North America and northern Europe	Edible	(Hills, 2008)
<i>Stropharia halophila</i>	Saprotrophic	Strophariaceae	Yellow cap colour, dark spore print, ring on the stem; Stipe whitish to pale yellowish, dry;	Foreshore dunes or outer related dunes, close to <i>Ammophila arenaria</i> ;	Inedible	(Guinberteau, Jahnke, Hoffmann and Pacioni, 1988)
<i>Tricholoma focale</i>	Mycorrhizal	Tricholomataceae	Cap sticky at first, later dry; covered with long fibrils; brown to yellow brown, orange-brown; Odour mealy;	Wooden dunes or close to <i>Pino-Quercetum ilicis</i> ;	Inedible	(Kalamees, 1821)

Botanical name	Ecology	Family	Characterization	Habitat	Edibility	References
<i>Tricholoma auratum</i>	Mycorrhizal	Tricholomataceae	Cap bright to olive brown or brownish centre; Stem pale yellow or whitish; odour mealy;	Pine forests (<i>Pinus pinaster</i>) of sea-side sand dunes;	Inedible	(Moukha et al., 2013)
<i>Tricholoma caligatum</i>	Mycorrhizal	Tricholomataceae	Small and fuscous to blackish fruit body, taste bitter;	Sand dunes, close to <i>Pinus nigra</i> ;	Inedible	(Murata et al., 2013)
<i>Tricholoma columbetta</i>	Mycorrhizal	Tricholomataceae	White to ceamy or bluish fruit body; odourless;	Deciduous and coniferous woods	Edible	(Vadalà et al., 2003)
<i>Melanoleuca cinereifolia</i>	Saprotrophic	Tricholomataceae	With a dark cap covering pallid gills; Cap, gills and stem all become ingrained with sand;	White dunes close to <i>Ammophila arenaria</i> ;	Inedible	(Guinberteau, Vizzini et al., 2012)
<i>Clitocybe odora</i>	Saprotrophic	Tricholomataceae	Pale greyish blue to greyish green, convex later expanded; strong anise odour	Soil, litter in deciduous and coniferous forests close to <i>Pino-Quercetum ilicis</i> ;	Edible	(Heinze, 2012)
<i>Amanita ovoidea</i>	Mycorrhizal	Amanitaceae	Cap convex to shield shaped; colour White to creamy;	Wooden dunes, sandy calcareous soil or close to <i>Quercus pubescens</i> ;	Edible	(Guinberteau)
<i>Cortinarius suberretorum</i>	Mycorrhizal	Cortinariaceae	Lemon yellow cap; body red to lightly;	Mediterranean close to oaks or <i>Pino-Quercetum ilicis</i> ;	Inedible	(Guinberteau)
<i>Cortinarius</i>	Mycorrhizal	Cortinariaceae	Brown spore mushroom;	Deciduous forests	Inedible	(Bronz et al., 2007)

Botanical name	Ecology	Family	Characterization	Habitat	Edibility	References
<i>infractus</i>			odour and taste may be explained of the indole alkaloids infraction;	with oak, beech, or chestnut from Mediterranean area;		
<i>Russula badia</i>	Mycorrhizal	Russulaceae	Cap red or black-purple-red colour, a stem of white, sometimes reddish;	Coastal pine forests on sand dunes;	Inedible	(Burlingham, 1944)
<i>Lactarius vinosus</i>	Mycorrhizal	Russulaceae	Cap surface smooth, slightly viscous; vinaceous red with more orange and darker zones; smell faintly fruity;	Calcareous soils close to <i>Pinus</i> ; Distribution mainly Mediterranean;	Edible	(Nuytinck and Verbeken, 2003)
<i>Phaeolus schweinitzii</i>	Saprotrophic parasitic	Polyporaceae	Caps are velvety to hairy when young; from bright yellow to brown with age;	Roots and heartwood of living conifers; dead or decaying woods;	Inedible	(Cedano et al., 2001)
<i>Inonotus hispidus</i>	Saprotrophic parasitic	Polyporaceae	Surface felty/hairy varying from yellowish to tobacco-brown, finally blackish and bristly	Usually on deciduous trees; distributed in North America, Central and Southern Europe	Inedible	(Pegler, 1964)
<i>Hydnellum ferrugineum</i>	Mycorrhizal	Bankeraceae	The surface of body velvety or prickly whitish with exuded reddish droplets; old specimens dark brown	Pine trees and other confers.	Inedible	(Ainsworth et al., 2010)

2.2. PREPARATION OF EXTRACTS

Mushrooms were ground in a Microfine mill (MF-10, IKA, Staufen, Germany) through a 1.5-mm sieve and extracted with four solvents. HPLC grade cyclohexane (C), dichloromethane (D), methanol (M) and deionized water (W) were used to fractionate soluble compounds from the mushrooms in ascending polarity by sequentially extracting 2-100g (depending on material availability) mushroom powder in a Soxhlet extractor for 5 h (*Fig. 2.1*). The samples were air dried after each solvent extraction and finally the residues were extracted with boiling water during 5 h constantly mixing in the Ikamag “RTC basic” magnetic stirrer (IKA Labortechnik, Staufen, Germany). Organic solvents were removed in a vacuum rotary evaporator RV 10 (IKA, Staufen, Germany), while water extracts were freeze-dried. The organic solvents residues were removed with nitrogen. All extracts were kept in a refrigerator until further analysis.

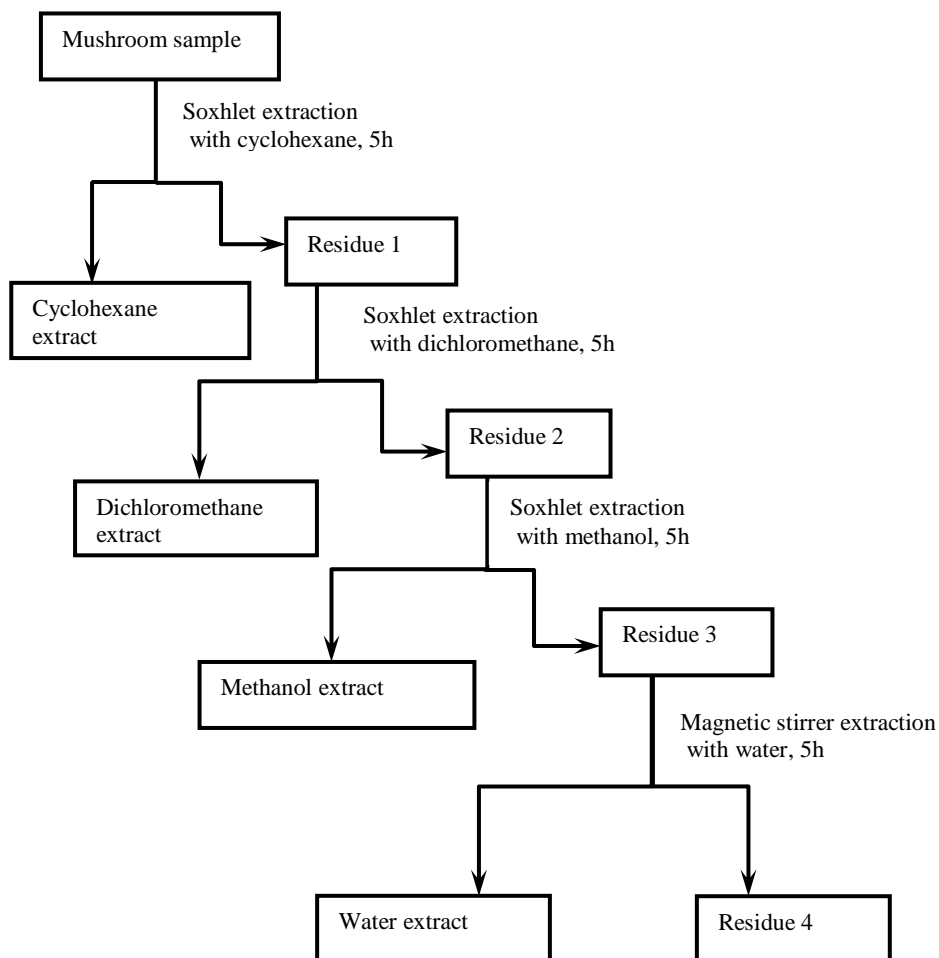


Fig. 2.1 Mushroom extraction scheme

2.2.1. Isolation of active compounds from *P. schweinitzii* extract

The methanol crude extract of *P. Schweinitzii* (2 g) was dissolved in methanol (50 mL), and added to silica gel Si 60 (2g, 230–400 mesh) by constant stirring. Methanol was evaporated in a rotary evaporator under vacuum at 45 °C. The fraction impregnated silica gel was cooled down to 25 °C and kept in a desiccator until required.

The silica gel chromatography column was packed as follows: the bottom of the chromatography column was plugged with glass wool to retain solids. Silica gel Si 60 (100g, 200–300 mesh, 40–63µm particle size) was suspended in dichloromethane (500 mL), and then transferred to the column (600 mm length × 30 mm i.d.) in which there was no trapped air. The column was rinsed with 500mL of dichloromethane, three times. As soon as the silica gel settled to the bottom of the column, sample impregnated silica gel was added to the top of the column, and elution was performed with a dichloromethane/methanol gradient (98:2, 95:5, 90:10, 85:15, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100%, 300mL each) at a constant flow rate of 8 mL/min. Fractions of 15 mL each were analyzed by TLC. TLC analyses were performed on silica gel F₂₅₄ plates using chloroform/methanol/water (65:45:10) as developing reagent. Spots were detected at 254 and 366 nm. The eluates containing target compounds were pooled and concentrated under reduced pressure. Fractions were purified on Sephadex LH-20 column (100 mm length × 10 mm i.d.) eluted with methanol. The concentrate was stored at –10 °C for further HPLC-preparative separation.

2.3. RESEARCH METHODS

Different methods were used for the analysis of mushroom extracts (Fig. 2.2) and bioactive compounds isolated from *P. schweinitzii* (Fig 2.3). All the methods are presented in schemes and further described in detail.

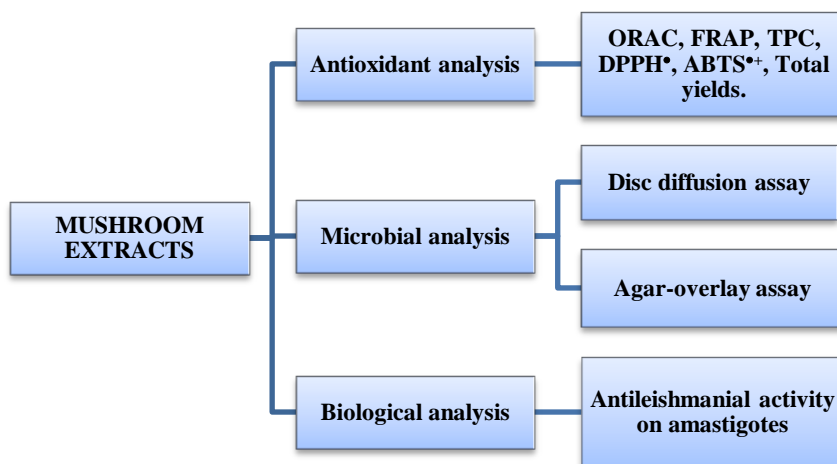


Fig 2.2 Scheme of analysis used for mushroom extracts

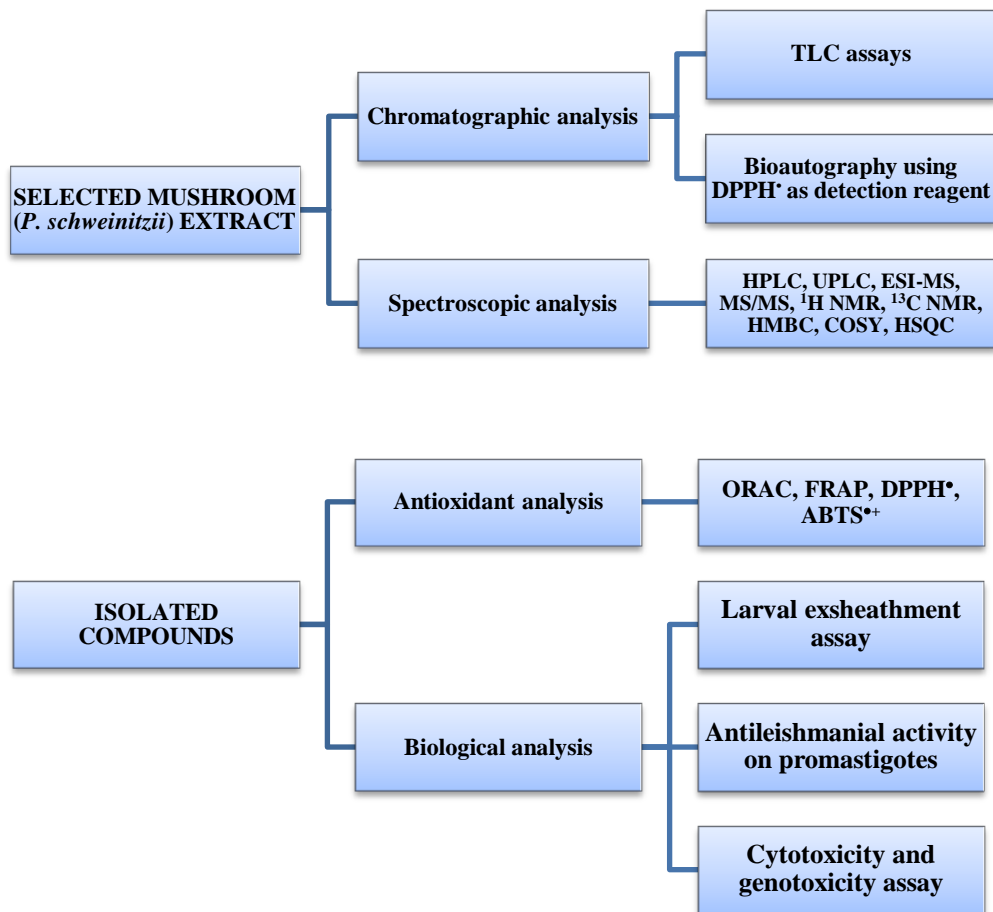


Fig 2.3 Schemes of analysis used for selected extract and isolated compounds from *P.schweinitzii*

2.3.1. ANTIOXIDANT ANALYSIS

2.3.1.1. DPPH[•]-scavenging capacity

This method is based on scavenging DPPH[•] by the antioxidant colorimetric changes (from deep violet – to light yellow), when DPPH[•] is reduced (Brand-Williams, Cuvelier and Berset, 1995). The assay was performed in a 96-well microtiter plates using an UV spectrophotometer EL×808 Microplate Reader (BioTex Instruments, Vermont, USA). The reaction mixture in each of the 96-wells consisted of 7.5 μL of different concentration mushroom extracts (0.5%; 0.25%; 0.125%) and 300 μL of methanolic solution of DPPH[•] (6×10^{-5} M). The mixture was left to stand for 40 min in the dark and the reduction of DPPH[•] was determined by measuring the absorption at 515 nm. All measurements were performed in triplicate. Radical scavenging capacity (RSC) was determined from the calibration curve,

which was drawn by using 50, 100, 125, 250, 500, 1000 $\mu\text{M/L}$ concentration solutions of Trolox and expressed in μM of Trolox equivalents (TE) per g dry extract weight ($\mu\text{M TE/g edw}$).

2.3.1.2 ABTS⁺ decolourisation assay

The Trolox equivalent antioxidant capacity (TEAC) assay is based on the scavenging of ABTS⁺ by the antioxidant which may be measured spectrophotometrically (Re et al., 1999). A stock solution of 2 mM ABTS was prepared by dissolving reagent in 50 mL of phosphate buffered saline (PBS) obtained by dissolving 8.18 g NaCl, 0.27 g KH_2PO_4 , 1.42 g Na_2HPO_4 and 0.15 g KCl in 1 L of Milli-Q water. If pH was lower than 7.4, it was adjusted with NaOH. ABTS⁺ was produced by reacting 50 mL of ABTS stock solution with 200 μL of 70 mM $\text{K}_2\text{S}_2\text{O}_8$ solution in purified water and allowing the mixture to stand in the dark at room temperature for 15–16 h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. For the assessment of extracts, the ABTS⁺ solution was diluted with PBS to obtain the absorbance of 0.800 ± 0.030 at 734 nm. One mL of ABTS⁺ solution was mixed with 10 μL extract solution in 96-well microtiter plates. The absorbance was read at ambient temperature every minute during 40 min. PBS solution was used as a blank; all measurements were performed in triplicate. The TEAC was determined from the calibration curve, which was drawn using 50, 100, 125, 250, 500, 1000 $\mu\text{M/L}$ concentration solutions of Trolox and calculated in $\mu\text{M TE/g edw}$ as follows:

$$TEAC \left(\frac{\mu\text{M}}{\text{g}} \right) = \frac{TE_s}{1000} \times \frac{V_s}{m_s},$$

TE_s – antioxidant activity of sample expressed in TE (μM), V_s – sample volume (mL), m_s – sample mass (g).

2.3.1.3. Ferric-reducing antioxidant power assay

Ferric-reducing antioxidant power (FRAP) assay is based on the reduction of Fe^{3+} in its tripyridyltriazine complex to the blue Fe^{2+} form (Benzie and Strain, 1999). The final results were expressed in $\mu\text{M TE/g edw}$. The FRAP reagent was prepared from acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily and was warmed to 37 °C in a water bath prior to use. Ten μL of sample were added to 300 μL of the FRAP reagent and 30 μL water. The absorbance of the reaction mixture was then recorded at 593 nm after 4 min. All measurements were performed in triplicate. The TEAC values were determined as indicated in previous sections.

2.3.1.4. Oxygen radical absorbance capacity

Oxygen radical absorbance capacity (ORAC) method was performed as described by (Prior, Wu and Schaich, 2005) and (Dávalos, Gómez-Cordovés and Bartolomé, 2004) by using fluorescein as a fluorescent probe. The reaction was carried out in a 75 mM phosphate buffer (pH 7.4); stock solution of fluorescein was prepared according to Prior et al (2005). Mushroom extracts were diluted 1:1000 (w/v); 25 μL of extract and 150 μL of fluorescein (14 μM) solutions were placed in

96 transparent flat-bottom microplate wells, the mixture was preincubated for 15 min at 37 °C and 26 µL of AAPH solution (240 mM) as a peroxy radical generator added with a multichannel pipette. The microplate was immediately placed in the FLUORstar Omega reader (BMG LABTECH, Ortenberg, Germany), automatically shaken prior to each reading and the fluorescence was recorded every cycle (66 s), totally 150 cycles. The 485-P excitation and 520-P emission filters were used. At least 4 independent measurements were performed for each sample. Raw data were exported from the Mars software to an Excel 2003 (Microsoft, Roselle, IL) sheet for further calculations. Antioxidant curves (fluorescence versus time) were first normalized and from the normalized curves the area under the fluorescence decay curve (AUC) was calculated as $AUC = 1 + \sum_{i=1}^{i=80} \frac{f_i}{f_0}$, where f_0 is the initial

fluorescence reading at 0 min and f_i is the fluorescence reading at time i . The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area under the curve (AUC). The TEAC values were determined as described in previous sections.

2.3.1.5. Determination of total phenolic content

The total phenolic content (TPC) was measured with Folin–Ciocalteu reagent as originally described by (Singleton, Orthofer and Lamuela-Raventós, 1999). Briefly, 30 µL (0.1%) of sample were mixed with 150 µL of 10-fold diluted (v/v) Folin–Ciocalteu reagent, and 120 µL of 7.5% Na₂CO₃. After mixing of all reagents, the microplate was placed in the reader and shaken for 30s. After incubation for 30 min at room temperature the absorbance of the mixtures was measured at 765 nm. All measurements were performed in triplicate. A series of gallic acid solutions in the concentration range of 0.025-0.35 mg/mL was used for the calibration curve. The results were expressed in mg of gallic acid equivalents per g of dry extract weight (mg GAE/g edw) and calculated by the following formula as $C = c \times \frac{V}{m}$, where C – the total content of phenolic compounds in mg/g mushroom extract, in GAE; c – the concentration of gallic acid established from the calibration curve in mg/mL; V – the volume of extract in ml; m – the weight of plant extract in g.

2.3.2. MICROBIAL ANALYSIS

2.3.2.1. Disc diffusion assay

The antimicrobial activity was assessed by the disk-diffusion method (Bauer et al., 1966). The bacterial cell suspension was prepared from 24h culture and adjusted to an inoculation of 1×10^8 colony forming units per mL (cfu/mL). Sterile nutrient agar was inoculated with bacterial cells (50µL of bacterial cell suspension in 25 mL medium) and poured into petri dishes to obtain a solid plate. Twenty mg of test material dissolved in the same solvent of the extraction were applied on sterile 5 mm diameter paper discs, which were deposited on the surface of inoculated agar plates. The plates with bacteria were incubated for 24 h at 37°C. Inhibition zone diameters

around each of the disc (diameter of inhibition zone plus diameter of the disc) were measured and recorded at the end of the incubation time. An average zone of inhibition was calculated from 3 replicates. Paper discs with solvents were used as controls.

Minimal inhibitory concentrations (MIC) showing the lowest concentration of extract able to inhibit any visible microbial growth was determined by the agar diffusion technique (Rajbhandari and Schöpke, 1999). The highest concentration of extract tested during the experiment was 20 mg/mL. The extracts were prepared at the series of concentrations (5; 10; 20 mg/mL) Ten μl of each concentration solution was transferred in the disk. Then the disks were transferred in the Petri dishes containing microorganism culture. The plates were incubated for 24 h at 37°C for bacteria. After incubation, the size of active zones of each concentration was measured. Each assay replicated three times. Paper discs with solvents were used as control.

2.3.2.2. Agar-overlay assay (TLC bioautography)

Thin-layer chromatography, combined with both biological and chemical detection methods, is an effective and inexpensive technique for the study of natural products. It can thus be performed both in sophisticated laboratories and in small laboratories which only have access to a minimum of equipment (Marston, 2011). When TLC is combined with a biological detection method, it is known as TLC bioautography. Historically, the technique of TLC bioautography has been known since 1946 (Goodall and Levi, 1946). This elegant, simple and rapid method is highly sensitive and permits immediate detection of biologically active compounds in the fractions, coupled with an accurate localisation of active substances. Bioautography are compact and simple tests which can be performed with a minimum of sample in a short time. The major applications of TLC bioautography are to be found in the fast screening of a large number of samples for bioactivity and in the target-directed isolation of bioactivity guided fractionations (Müller et al., 2004, Choma and Grzelak, 2011).

Bioautography assay was carried out for the mushrooms methanol fractions according to method described by (Sawaya et al., 2004) and (Mendonça-Filho, 2006). In the first step, 20 μL of mushrooms extracts (concentration 10mg/mL in methanol) were applied to TLC plates as a spot at a distance from 1.5 cm of the lower edge of the plate. The mobile phase was chloroform/methanol/water (65:45:10, v/v). Bioautography was carried out after airing the TLC plates for over 8 hours. The plates were covered with 20ml of sterile nutrient agar at 45°C inoculated with the microorganisms and then incubated for 24 hours at 37°C. After this period each plate was covered with 7ml of a 0.08% aqueous solution of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) and incubated for up to 24 hours at 37°C (Fig.2.4). Inhibition zones were visualized and recorded as clear areas against a violet coloured background. Each fraction replicated three times. TLC pates with solvent were used as control.

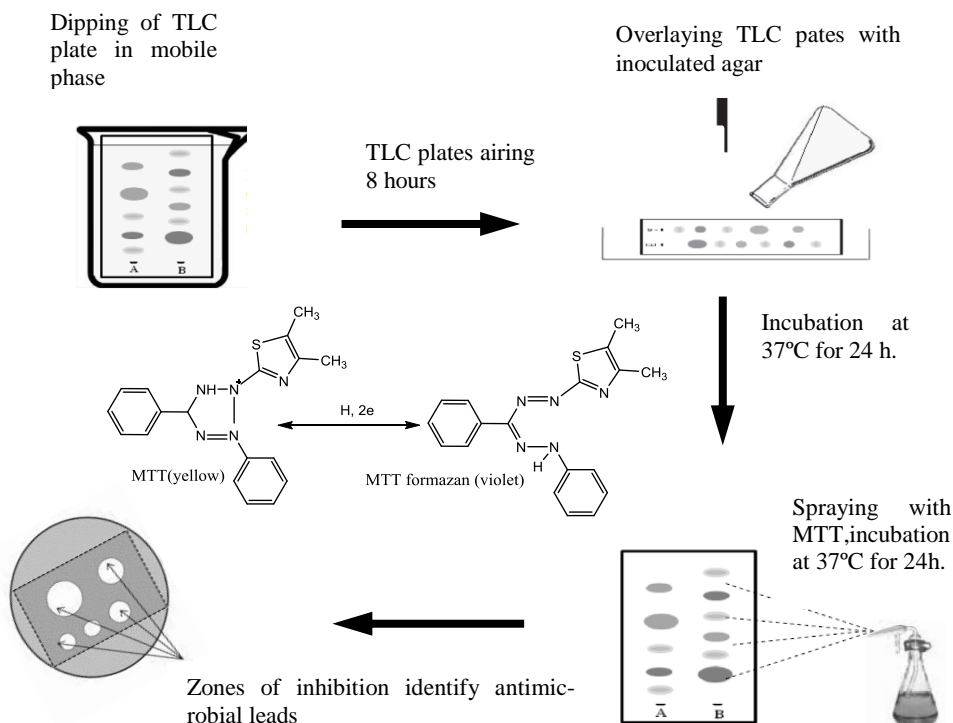


Fig. 2.4 Bioautography assay technique with microorganisms

2.3.3. BIOLOGICAL ANALYSIS

2.3.3.1. Antileishmanial activity on amastigotes of wild mushrooms species

For assessing the biological activity of mushroom fractions against the amastigote stage of the parasite, *Leishmania infantum* was used. Amastigotes were incubated at an average density 10^6 parasites/mL in a sterile 96-well plate with various concentrations of fractions dissolved in DMSO serially from 100, 10, 1, and 0.1 $\mu\text{g/mL}$. Appropriate controls treated by DMSO and amphotericin B reference drug were added to each set of experiments. After 72 h of incubation, 10 μL of MTT (10^{-3} mL/L) was added to each well and the plates were further incubated for 4 h. After the enzymatic reaction was stopped with 100 μL of a 50% isopropanol and 10% sodium dodecyl sulphate solution and the plates were incubated for an additional 30 min under agitation at 25°C temperature. The absorbance was measured at 595 nm with microplate Bio-Rad FTS-7 spectrophotometer (California, USA). Inhibitory concentration 50% (IC_{50}) was defined as the concentration of drug required to inhibit by 50% the metabolic activity of *Leishmania infantum* amastigotes compared to the control. IC_{50} were calculated by non-linear regression analysis processed on dose–response curves, using Excel 2010 software. IC_{50} values represent the mean value calculated from three independent experiments.

2.3.3.2. Antileishmanial activity on promastigotes of isolated compounds

The effects of the tested compounds on the growth of *Leishmania infantum* promastigotes were assessed by Luciferase Assay. Briefly, promastigotes in log-phase in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and antibiotics (100U/mL penicillin, 100 µg/mL streptomycin and 50µg/mL geneticin), were incubated at an average density of 106 parasites/mL in sterile 96-well plates with various concentrations of compounds dissolved in DMSO (final concentration less than 0.5% v/v), in duplicate. Appropriate controls treated by DMSO and amphotericin B were added to each set of experiments. After a 72h incubation period at 24°C, each plate-well was then microscope-examined for detecting possible precipitate formation. To estimate the luciferase activities of promastigotes, 80 µl of each well are transferred in white 96-well plates, Steady Glow reagent was added according to manufacturer's instructions, and plates were incubated for 2 min. The luminescence was measured in Microbeta Luminescence Counter (PerkinElmer, Connecticut, USA). Inhibitory concentration 50% (IC₅₀) was defined as the concentration of drug required to inhibit by 50% the metabolic activity of *Leishmania infantum* promastigotes compared to the control. IC₅₀ were calculated by non-linear regression analysis processed on dose–response curves, using Table Curve 2D V5 software. IC₅₀ values represent the mean value calculated from three independent experiments.

2.3.3.3. Cytotoxicity assay

The evaluation of the tested molecules cytotoxicity by MTT assay on the J774A.1 mouse macrophage cell line was done according to (Mosmann, 1983) with slight modifications. Briefly, cells (5.104 cells/mL) in 100 µL of complete medium, [RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and antibiotics (100U/mL penicillin and 100 µg/mL streptomycin)] were seeded into each well of 96-well plates and incubated at 37 °C in a humidified 6% CO₂ with 95% air atmosphere. After a 24 h incubation, 100 µL of medium with various product concentrations and appropriate controls were added and the plates were incubated for 72 h at 37 °C. Each plate-well was then microscope examined for detecting possible precipitate formation before the medium was aspirated from the wells. 100 µL of MTT solution (0.5 mg/mL in RPMI) were then added to each well.

Cells were incubated for 2 h at 37 °C. After this time, the MTT solution was removed and DMSO (100 µL) was added to dissolve the resulting formazan crystals. Plates were shaken vigorously (300 rpm) for 5 min. The absorbance was measured at 570 nm with a microplate spectrophotometer (PerkinElmer, Connecticut, USA). DMSO was used as blank and doxorubicin as positive control. The cytotoxic effect were expressed as 50% lethal dose (CC₅₀), i.e., as the concentration of a fraction which reduced 50% of cell viability compared to cell in culture medium alone. CC₅₀ were calculated by non-linear regression analysis processed on dose–response curves, using Excel 2010 software. CC₅₀ values represent the mean value calculated from three independent experiments. The selectivity index (SI) for each compound was

calculated as ratio between, cytotoxicity (CC₅₀) and activity (IC₅₀) against *Leishmania infantum* amastigotes and promastigotes.

2.3.3.4. Larval exsheathment assay

This test uses ruminant gastrointestinal nematodes (infective third stage) in a two-stage process aimed at examining the effect of the test product on larval exsheathment induced by a diluted sodium hypochlorite solution. This is an adaptation process and the aim is to obtain a progressive exsheathment of the larvae, making microscopic observation of the process feasible. For a control, a 100% exsheathment after 60 min is observed; negative controls (PBS) and tested substances are incorporated into the assay (Jackson and Hoste, 2010). 1300 L₃/mL ensheathed *H. contortus* L₃ were incubated with each compound at a concentration of 300, 150, 75, 37.5 µg/mL in PBS for 3 h at 21 °C. For control larvae incubated in PBS. After incubation, the larvae were washed and centrifuged (1000 rpm) three times in PBS (pH 7.2). Larvae were then subjected to an artificial exsheathment process by contact with a solution of sodium hypochlorite (2%, w/v and the 16.5% w/v sodium chloride) solution diluted 1:300 in PBS (pH 7.2). The kinetics of the larval exsheathment process in each experimental treatment was monitored by microscopic observation (x200 magnification). Exsheathed larvae were counted at 0, 20, 40, 60 min. At each time the larvae were killed and examined immediately. Four replicates were run for each compound to look for possible changes in the proportion of exsheathed larvae over time. A general linear model (GLM) test was used to determine the difference in the mean percentage of exsheathment rates between the control and the treatment groups over time: $(\%) = \frac{N_{ex}}{N} \times 100$; Ex – Exsheathment (%); N_{ex} – exsheathed larvae; N – total larvae.

2.3.3.5. Detailed biological activity assays of hispidin

2.3.3.5.1. Cell culture and cytogenetic procedures

Lymphocyte cultures from three healthy donors were set up according to conventional techniques within 2-4 h after blood collection. In brief, phytohemagglutinin (PHA, 7.8 µg/mL) stimulated cultures were incubated at 37°C for 72 hours in RPMI 1640 medium supplemented with 12% heat-inactivated newborn calf serum, 40 µg/mL gentamycin. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Treatment with hispidin was carried out 24 h after culture initiation and lasted for the period of 48 h. Hispidin was dissolved in ethanol and then diluted with RPMI 1640 medium to the desired concentration. Ethanol in a final concentration of 7.5µL/mL was used as a solvent control. Ethanol concentration in the experimental series did not exceed this concentration. Working solutions were made just before treatment. Two parallel cultures were used for each concentration of the compound tested.

2.3.3.5.2. Cytokinesis-block micronucleus assay

Lymphocytes from two donors (donor F1 - female, 22 years-old, and donor M1 - male, 27 years-old) were used in this part of study. In the cultures assigned to micronucleus analysis cytochalasin B at a final concentration of 6 µg/mL (Sigma-Aldrich, St. Louis, MO, USA) was added to the cultures 44 h after PHA stimulation to arrest cytokinesis and induce binuclear cell formation. At 72 h, the cells were treated hypotonically with 0.075 M KCl, fixed with methanol:acetic acid (5:1, v/v), air-dried and stained with Giemsa (5% in Soerensen's buffer). All slides were coded and analyses were performed on coded slides by a single scorer at 1000× magnification on a light microscope (Nikon Eclipse 80i, Japan). The presence of micronuclei (MN) was evaluated by scoring a total of 1000 binucleated (BN) cells for each donor following the scoring criteria of Fenech (Fenech, 2007). The nuclear division index (NDI) was used for measuring cell proliferation kinetics and was calculated from 500 cells according to the formula: $NDI = (M_1 + 2M_2 + 3M_3 + 4M_4) / N$, where M_1 , M_2 , M_3 and M_4 represent the number of cells with one, two, three and four nuclei and N is the total number of cells scored.

2.3.3.5.3. Chromosome aberration and sister chromatid exchange assay

Blood sample obtained from one donor (donor F2 - female, 22 years-old) was used for this experiment. Cultures were treated with 10 µg/mL 5-bromo-2'-deoxyuridine (BrdU) for the entire culture period and colchicine (0.6 µg/mL) for the last 3 hours of incubation. The cells were harvested, hypotonically swollen in 0.075 M KCl (25 min) and fixed in methanol : acetic acid (3 : 1) fixative with three changes. Air-dried slides were differentially stained by fluorescence plus Giemsa technique. Briefly, the slides were stained for 10 min. with 10 µg/mL of Hoechst 33258 dye (dissolved in 0.07 M Soerensen's buffer, pH 6.8). Then the slides were rinsed, mounted with citrate buffer (pH 8.5), covered with cover slips and exposed to UV light (400 W mercury lamp at a distance of 15 cm) for 6-7 min. Slides were then rinsed and stained for 3-4 min. with 5% Giemsa. Sister chromatid exchange (SCEs) were scored by a single scorer in no less than 50 second-division metaphases per treatment. Two hundred cells were scored for the cell replicative kinetics determined by means of replication index RI ($RI = [M_1 + 2M_2 + 3M_3] / N$, where M_1 , M_2 , M_3 are the numbers of cells that had undergone one, two or three cycles of replication, and N is the total number of cells scored). No less than 200 first-division metaphases per concentration were analysed for chromosome aberrations. The CA were recorded according to An International System for Human Cytogenetic Nomenclature (Shaffer and Tommerup, 2005). Aberrations were scored as individual types (chromatid and chromosome type breaks and exchanges), but for statistical analysis only total CA per 100 cells was used.

2.3.3.5.4. Comet assay

The comet assay was conducted under alkaline conditions according to Singh et al. (Singh et al., 1988) with minor modifications. Peripheral blood was obtained immediately before the conducting the test from two healthy donors (donor F1 and

donor F3 - female, 22 years-old) into heparinized vacutainer tubes (Becton-Dickinson, USA) by venipuncture. Lymphocytes were isolated by Lymphoprep density gradient centrifugation according to the manufacturer's instructions (Axis-Shield, Norway). Following isolation, the cells were resuspended in RPMI 1640 medium and incubated with increasing concentrations (dose range from 25 to 1000 μM) of hispidin for 1 h at 37°C in a 5% CO_2 . Hispidin solutions were prepared just before treatment. Negative (ethanol, 3% v/v), blank and positive (20 μM H_2O_2) controls were also included at the same temperature and exposure time. Following incubation, the lymphocytes were centrifuged at 400 x g for 10 min., the supernatant was then drawn and the cells were resuspended in RPMI 1640 medium. Thereupon, 40 μl of cell suspension was mixed with 40 μl of 1% low melting point agarose (LMP), pipeted onto the slides precouted with 1% normal melting point agarose (NMA) and covered with a coverslip. The agarose was allowed to solidify for 10 min. at 4°C. The coverslip was then removed, and the next layer of 0.5% LMP agarose was added, covered with a coverslip and left to solidify for additional 10 min. at 4°C. After the coverslips were taken off, the slides were immersed in cold freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2EDTA , 10 mM Tris, with 1% Triton X-100 and 10% DMSO added just before use, pH 10) in the dark for 1.5 hour at 4°C. Then the slides were removed from the lysis solution, and placed in a horizontal gel electrophoresis tank filled with ice-cold fresh electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH, pH 13). The slides were left in the solution for 20 min to facilitate DNA unwinding, and then electrophoresis was carried out at 17 V and 300 mA for 30 min. After electrophoresis, the slides were neutralized with Tris buffer (0.4 M Tris, pH 7.5), and stained with 80 μL ethidium bromide (20 $\mu\text{g}/\text{mL}$). All the above steps were conducted under dimmed light to prevent additional DNA damage. Low melting point agarose, Na_2EDTA , Tris, Triton X-100 was from Carl Roth GmbH (Germany). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA). The slides were examined by a single scorer using a fluorescence microscope (Nikon Eclipse 80i, Japan) at 400 x magnification. Image capture and analysis were performed using *Lucia Comet Assay* Software. In all cases 100 comets were scored and percentage of DNA in the comet tail (% TDNA) was used as DNA damage parameter.

2.3.3.5.5. Salmonella/microsome test

Plate incorporation method using histidine-dependent *S. typhimurium* TA98 and TA100 strains in the presence and absence of metabolic activation system (S9 liver fraction) was used for assessing the mutagenicity (Mortelmans and Zeiger, 2000). Moltox (Trinova Biochem, Germany) products for Ames test were used in the present study. Hispidin was tested at four concentrations (dose range from 50 to 250 $\mu\text{g}/\text{mL}$). 100 μL of extract solution were added to 2 mL top agar mixed with 100 μL of bacterial culture and then poured on to a plate containing minimal glucose agar. These plates were incubated at 37°C for 48 h and revertant colonies were counted using automatic Sorcerer colony counter (Perceptive Instruments, UK). The influence of metabolic activation was tested by adding 500 μL of S9 mixture. The

experiments were analysed in triplicate and the results are presented as the mean of the three plates with standard deviation. The criteria employed to interpret the results of Ames test as positive were similar to those used in regulatory guidelines (OECD.). The number of induced mutations should be at least twice the activity observed in negative control and there must be a reproducible dose response curve. Concurrent positive and negative (ethanol, 3% v/v) controls were used in the study. The standard mutagens used as positive controls were daunomycin for TA98 strain and sodium azide for TA100 strain without metabolic activation and 2-aminoanthracene for both TA98 and TA100 strains with metabolic activation.

2.3.4. CHROMATOGRAPHIC ANALYSIS

2.3.4.1. Thin layer chromatography on selected mushroom extract

The 5 μ l methanol solution of *P. schweinitzii* (10mg/mL) was directly deposited on three silica gel G60 F₂₅₄ plates. Chloroform/methanol/water (65:45:10) were used as mobile phase to develop thin layer chromatography (TLC) plates. The TLC plates were placed in solvents chamber until the developing reagents reach front 1cm. from the top of plate. The developed TLC plates were removed from the solvents, and allowed to air-dry. UV active absorbing spots were detected at 254 and 366 nm and exposing with adequate TLC reagents vapor: Vanillin/sulfuric (100 mL:1g vanillin, 10mL sulfuric acid, 90mL ethanol, 95%) reacts as universal with many compounds such as terpenoids, sterols and alkaloids and in general with lipophilic compounds forming dark-coloured zones; Neu (100mL: 1g 2-aminoethyl diphenylborinate, 5g polyethylene glycole, macragol 4000, 100mL ethanol 95%) reacts with phenolic compounds showing hydroxyl groups such as flavonoids, sugars, anthocyanines or hydroxyl acids; Dragendorff's reagents (Solution A : 0.8g bismuth nitrate, 10mL glacial acetic acid, 50mL water ; Solution B: 20g potassium iodide, 50mL water; Solution C: 5mL solution A and 5mL solution B, 50mL distilled water) identify nitrogen-containing compounds such alkaloids class compounds; These reagents identified the category of active secondary metabolite - phytoconstituent of *P. schweinitzii* extract.

2.3.4.2. Bioautography using DPPH[•] as detection reagent

P. schweinitzii methanol extract was tested for their antioxidant activities based on DPPH[•] scavenging capacity, using a TLC bioautography method (Marston, 2011, Cieřla et al., 2012). The 5 μ L methanol solution of *P. schweinitzii* (10mg/mL) was directly deposited on silica gel G60 F₂₅₄ plates. Chloroform/methanol/water (65:45:10) mixture was used as mobile phase to develop TLC plates. The TLC plates were placed in solvents chamber until the developing reagents reach front 1cm. from the top of plate. The developed TLC plates were removed from the solvents, and allowed to air-dry for 30min, followed by spraying with 0.1% w/v DPPH[•] methanol solution for activity identification. Bands with the DPPH[•] scavenging activity were observed as white yellow bands on a purple background. Experiment was replicated three times.

2.3.4.3. HPLC-preparative chromatography

The HPLC (High performance liquid chromatography) autopurification system applied consisted of Waters 2767 sample manager (Waters, Milford, MA, USA) equipped with a Waters 2489 UV/Vis detector, 2545 binary gradient module, system fluidics organizer and MassLynx Software with the FractionLynx application manager. The chromatographic separation was carried out using analytical and preparative XBridge 5- μm (in 4.6 i.d. \times 150 and 19 i.d. \times 150 respectively) mm columns at 25 °C. The mobile phase consisted of 0.02% (v/v) formic acid solution in water (solvent A) and acetonitrile (solvent B). The optimized linear gradient elution program was determined to be 0–40% solvent B at 0–0.5 min, 40–100% solvent B at 0.5–12.50 min, 100% solvent B at 12.5–14.50 min, 100–40% solvent B at 14.50–15 min. The flow rate was set at 20 mL/min and the injection volume of sample was 250 μL .

2.3.4.4. UHPLC- analytical chromatography

The UHPLC (Ultra high performance liquid chromatography) Acquity system combined with PDA (photo diode array detector) type eLambda 800nm and equipped with SQD 4271 (simple quadrupole detector), (Waters, Milford, MA, USA). UHPLC have binary solvent delivery system and autosampler with a 2.0 μL sample loop. An Acquity (Waters) CSH C18 column (1.7 μm , 50 \times 2.1 mm, i.d.) was used for separation of compounds. The mobile phase was initially composed of 98% eluent A (0.02% v/v formic acid solution in ultra pure water) and 2% B (0.02% v/v formic acid with acetonitrile), followed by a linear gradient: 2% solvent B at 0–2.4 min, 98% solvent B at 2.4–3.0 min, 2% solvent B at 3.0–3.50 min, temperature 35 °C. The flow rate was set at 0.6 mL/min and the injection volume of sample was 1.0 μL and the effluent was monitored at 377nm. The effluent from the PDA detector was introduced directly into SQD mass spectrometer and data acquisition were observed with Empower 2 Build 2154 software. MS experiments were performed in positive ionization mode.

2.3.5. STRUCTURE ELUCIDATION OF ISOLATED COMPOUNDS

The structures of the purified compounds were elucidated by various spectroscopic methods: UPLC-PDA-SQD, UV, ESI-MS, ESI-MS/MS, 2D NMR, ^1H and ^{13}C NMR. Acquity UPLC equipped with PDA (photo diode array detector) – SQD (simple quadrupole detector), type eLambda 800nm (Waters, Milford, MA, USA). ^1H NMR spectra were recorded on a Bruker Avance 500 (Bruker, Rheinstetten, Germany). ^{13}C and 2D spectra (HMBC, COSY and HSQC) were recorded on a Bruker DPX 400. Mass spectra were recorded on a UPLC Xevo G2 Q TOF or UPLC/ESI-QTOF-MS (Waters, Milford, MA, USA).

2.4. STATISTICAL ANALYSIS

Mean values and standard deviations (SD) of antioxidant, antimicrobial and cytotoxic activity from all experiments were performed at least three replications (n=3). RSC, FRAP, ORAC, and TPC values were calculated with n=4 replicate

measurements as indicated in each section. Statistical analysis of the obtained results was performed by using one-way analysis of the variance (ANOVA), the differences between samples showed significant variation ($p < 0.05$). Correlation coefficients (R) to determine the relationship between two variables, RSC, FRAP, ORAC and TEAC tests were calculated using MS Excel 2010 software (CORREL statistical function).

The antioxidant indicators of extracts were summed-up by using integrated values – ‘antioxidant score’ of extract (ASE), which is the sum of values for the fraction obtained with the same solvent in all assays. ASE is expressed in the so-called ‘comparative integrated units’ in 1 g of dry extract weight (ciu/g edw) and for the whole mushroom dry material, expressed in 1 g of dry mushroom weight (ciu/g mdw). The latter values, reflecting ‘antioxidant scores of mushrooms’ (ASM) take into the account ASE and extract yields (EY); they were calculated as follows: $ASE_c \times EY_c / 100 + ASE_d \times EY_d / 100 + ASE_m \times EY_m / 100 + ASE_w \times EY_w / 100$. These integrated values to some extent reflect the total antioxidant potential of the tested mushroom species, which consider extract yields, as well as the effectiveness of different polarity solvents used for the extraction. The concept of antioxidant scores may assist in assessing a large number of antioxidant activity data obtained in this study.

Statistical analysis for detailed biological assays of hispidin was performed using the SPSS (Version 19) package (SPSS, Inc., Chicago, IL, USA). Statistical tests were chosen according to the nature of the data analysed. The Student’s two sided *t*-test was applied for MB, CA, SCE and comet assay results, comparing the different treatment groups, and *z* test for NDI and RI analysis (Lazutka, 1991). Dose–response relationships were determined by Pearson’s correlation. *P* value < 0.05 was regarded as statistically significant.

3. RESULTS AND DISCUSSION

3.1. YIELDS AND ANTIOXIDANT PROPERTIES OF WILD MUSHROOM EXTRACTS

Antioxidants with the source of healthy compounds are present in all biological systems and mushrooms are not exception. There are developed a large number of assays for evaluating antioxidant capacities of botanical extracts. Following the scientific recommendations (Huang et al., 2005) all these methods were applied for the comprehensive assessment of antioxidant potential of the obtained with different solvents mushroom extracts. To obtain comparable values the results of ABTS^{•+}, DPPH[•], FRAP and ORAC assays were expressed in Trolox (a hydrosoluble analogue of vitamin E) equivalents, i.e. in the amount of Trolox μM possessing similar antioxidant capacity as 1 g edw, while TPC was expressed in mg of gallic acid equivalents per g of edw (*Table 3.2*). Remarkable diversity in antioxidant capacity, depending on mushroom species, extraction solvent and assay method, may be clearly observed. Therefore, it is convenient to consistently discuss the effect of these factors in separate sections. For easier assessment of antioxidant potential of different mushroom species, as well as the effects of different solvents and applied antioxidant activity assays (*Table 3.2*). The antioxidant characteristics were also summarized by using ‘antioxidant scores’ of extracts (ASE) and dry mushroom material (ASM) and expressed in the so-called ‘comparative integrated units’ (ciu), which are explained in 2.4 section.

To the best of our knowledge such approach is applied for the selected mushroom species for the first time. Moreover, there are not any available literature sources about testes species, except the rough reports on antioxidant properties of *A. devoniensis*, *B. impolitus* and *C. odora*.

3.1.1. Yield of mushroom extracts fractionated with different solvents

Efficient extraction of antioxidants and other biologically active molecules requires the use of solvents with different polarities: certain antioxidants are better soluble in polar solvents such as methanol, water, while cyclohexane or dichloromethane are preferable for isolating lipophilic compounds. Two main approaches may be applied for exhausting isolation of various components from biological material, namely parallel extraction of initial material with different solvents or sequential fractionation with increasing polarity and dielectric constant solvents. The latter approach was applied in our study: non-polar cyclohexane was followed by polar aprotic solvent dichloromethane; the residues were further extracted with polar protic solvent methanol and the process for several species was finalized with boiling water possessing the highest dielectric constant. It is obvious (*Table 3.1*) that selected mushrooms are composed of very different classes of substances from the point of view of their solubility in the applied solvents. The yields of total mushroom species sorted by families represented in *Fig. 3.1*. Dominated families with possessed significant total yields were Agaricaceae, Tricholomataceae, Boletaceae. The yield of non-polar cyclohexane-soluble fraction

depending on mushroom species was from 0.61 % (*S. halophila*) to 5.14 % (*R. badia*), aprotic solvent dichloromethane yielded from 0.52 % (*G. ammophilus*) to 3.42 % (*T. caligatum*), protic methanol from 6.77 % (*S. halophila*) to 41.29 % (*A. pseudoprattensis*), more hydrogen bonding components. While boiling water from 6 species additionally extracted from 2.94 % (*H. ferrugineum*) to 25.29 % (*T. caligatum*) of hydrosoluble components. Thus, the highest total yield of all fractions was obtained from *T. caligatum* (63.15%), while the lowest one from *S. halophila* (8.54 %). Protic solvents possessing high dielectric constant gave remarkably higher extract yields comparing with non-polar and aprotic solvents. It proves that all tested mushroom species contain low amounts of lipophilic constituents. The yields obtained by different solvents are very important characteristics in applying biorefinery concept to biomaterials for their effective, preferably no-waste conversion into the fractions for different applications. For comparison, previously a reported yield of methanol extracts of *B. lupinus* was 37.67% (Nikolovska-Nedelkoska et al., 2013); thus, it is similar to the sum of extracts (36.01%) obtained in our study for this species; however, in reported studies the initial material was extracted. Sequential extraction of *A. devoniensis* with dichloromethane, methanol and water extracted at room temperature was also applied previously and the yields were 5.3, 11.5, and 18.3% respectively (Al-Fatimi et al., 2005) In our study dichloromethane yield was lower (1.37%) while methanol yield (33.01%) was remarkably higher. The yields of ethanolic and water soluble polysaccharide fractions of *C. odora* under agitation extraction were reported 1.26 and 18.68% (Vaz et al., 2011); thus the sum of extracts obtained in their study (19.94%) was lower than the sum of yields (35.43%) obtained in our work for this species. The yield of phenolic and polysaccharide fractions of *X. chrysenteron* from Portugal were reported 12.28 and 27.40% (Heleno et al., 2012); thus, the sum of extracts (39.68%) is similar to the sum of yields (39.93%) obtained in our study for this species. More than half tested species have higher sum of yields than well-known edible species “Paris mushroom” (*A. bisporus*) sequentially extracted with hexane, ethyl acetate and aqueous methanol (7.17%) or “oyster mushroom” (*Pleurotus ostreatus*) methanol (16.9%) or (12.01%) ethanol fractions (Öztürk et al., 2011, Yang, Lin and Mau, 2002, Arbaayah and Kalsom, 2013).

Table 3.1 The yields of mushroom extracts isolated by different solvents, % (w/w)

Botanical name	C	D	M	W	Total
<i>A. menieri</i>	2.40*	0.67*	27.08*	◆	30.15
<i>A. pseudoprattensis</i>	3.00*	1.72*	41.29*	◆	46.01
<i>A. freirei</i>	1.47*	0.90*	34.50*	◆	36.87
<i>A. devoniensis</i>	2.28*	1.37*	33.01*	◆	36.67
<i>A. fissuratus</i>	3.43*	0.89*	26.37*	◆	30.69
<i>A. coniferarum</i>	1.22*	1.06*	9.90*	◆	12.18
<i>L. litoralis</i>	2.61*	0.87*	29.70*	◆	33.19
<i>L. subolivaceus</i>	1.66*	0.75*	26.49*	◆	28.90
<i>S. subvolvatus</i>	2.15*	0.89*	34.63*	◆	37.76
<i>G. ammophilus</i>	1.20*	0.52*	17.77*	◆	19.50

Botanical name	C	D	M	W	Total
<i>B. Lepidus</i>	3.57*	1.16*	22.78*	◆	27.45
<i>B. implitus</i>	1.87*	1.04*	15.55*	◆	18.46
<i>B. luridus</i>	1.56*	0.54*	21.01*	◆	23.11
<i>B. lupines</i>	1.18*	1.45*	33.39*	◆	36.01
<i>X. chrysenteron</i>	2.64 ± 0.25	1.90 ± 0.20	14.16 ± 0.12	21.23 ± 0.17	39.93
<i>S. halophile</i>	0.61*	1.16*	6.77*	◆	8.54
<i>T. focale</i>	2.63*	0.75*	24.54*	◆	27.91
<i>T. auratum</i>	4.39*	0.75*	31.44*	◆	36.58
<i>T. caligatum</i>	4.00 ± 0.13	2.79 ± 0.10	36.69 ± 0.25	19.67 ± 0.30	63.15
<i>T. columbetta</i>	1.55 ± 0.05	1.57 ± 0.30	18.74 ± 0.30	25.29 ± 0.25	47.15
<i>M. cinereifolia</i>	3.17*	0.86*	19.57*	◆	23.60
<i>C. odora</i>	2.95*	1.03*	31.45*	◆	35.43
<i>A. avoidea</i>	3.52*	1.61*	35.58*	◆	40.71
<i>C. suberretorum</i>	3.69*	1.58*	27.37*	◆	32.64
<i>C. infractus</i>	3.03*	1.24*	29.98*	◆	34.25
<i>R. badia</i>	5.15*	1.05*	33.23*	◆	39.42
<i>L. vinosus</i>	4.99*	1.06*	25.59*	◆	31.64
<i>P.schweinitzii</i>	3.75 ± 0.30	2.07 ± 0.16	13.91 ± 0.20	4.82 ± 0.20	24.55
<i>I. hispidus</i>	0.40 ± 0.15	0.43 ± 0.25	4.55 ± 0.10	11.10 ± 0.15	16.48
<i>H. ferrugineum</i>	1.42 ± 0.12	1.33 ± 0.12	11.67 ± 0.16	2.94 ± 0.23	17.36

C, cyclohexane; D, dichloromethane; M, methanol; W, water; Results expressed: mean ± SD (n=3)

*Standard deviation not calculated, smples deficiency; ◆ sample not tested.

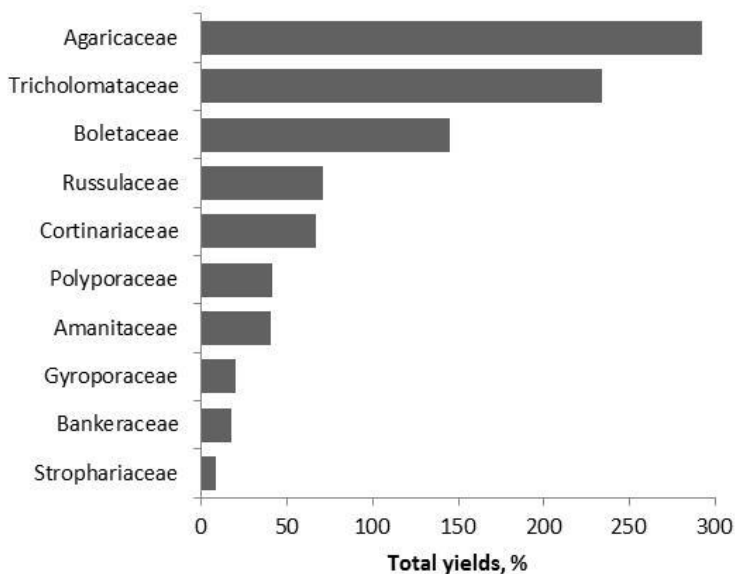


Fig.3.1 The total yields of mushroom extracts sorted by families

Table 3.2 Antioxidant activity of extracts isolated from tested mushroom species by different solvents.

Botanical name	Extract	$\mu\text{M TE/g edw}$				TPC, mg GAE/g edw
		ABTS ⁺	DPPH [•]	FRAP	ORAC	
<i>Agaricus menieri</i>	C	80.6 ± 2.8	10.5 ± 4.8	119.0 ± 6.0	45.8 ± 4.6	10.33±1.05
	D	344.0 ± 5.0	158.0 ± 2.9	331.0 ± 3.8	60.0 ± 4.9	14.29±0.62
	M	241.0 ± 4.8	95.5 ± 4.1	274.0 ± 4.0	54.1 ± 3.9	10.67±0.66
	Total	665.6	264.0	724.0	159.9	35.29
<i>Agaricus pseudoprattensis</i>	C	11.6 ± 4.4	48.0 ± 4.1	142.0 ± 3.8	83.8 ± 5.0	5.54±0.13
	D	67.6 ± 3.0	59.3 ± 4.8	212.0 ± 4.6	105.0 ± 4.4	8.41±0.09
	M	204.0 ± 1.9	69.3 ± 4.8	162.0 ± 5.0	117.0 ± 3.0	6.26±0.12
	Total	283.2	176.6	516.0	305.8	20.21
<i>Agaricus freirei</i>	C	147.0 ± 3.4	30.5 ± 4.1	181.0 ± 3.2	106.0 ± 1.8	9.88±0.76
	D	130.0 ± 4.3	23.0 ± 2.9	280.0 ± 4.6	156.0 ± 2.9	10.97±0.91
	M	358.0 ± 4.3	61.8 ± 4.8	364.0 ± 3.8	132.0 ± 4.6	7.34±0.10
	Total	635.0	115.3	825.0	394.0	28.19
<i>Agaricus devoniensis</i>	C	66.3 ± 4.2	27.2 ± 2.9	89.0 ± 4.6	9.9 ± 2.4	4.57±0.17
	D	54.6 ± 2.0	31.8 ± 2.9	188.0 ± 3.8	36.8 ± 4.7	4.46±0.16
	M	228.0 ± 3.1	65.5 ± 5.0	329.0 ± 3.3	56.5 ± 1.8	6.14±0.13
	Total	348.9	124.5	606.0	103.2	15.17
<i>Agaricus fissuratus</i>	C	26.6 ± 4.0	30.5 ± 2.8	113.0 ± 4.0	1.7 ± 1.0	5.26±0.12
	D	219.0 ± 4.2	24.7 ± 3.8	118.0 ± 3.8	8.0 ± 0.7	7.34±0.29
	M	229.0 ± 5.0	70.5 ± 5.0	289.0 ± 4.6	27.1 ± 4.7	6.22±0.10
	Total	474.6	125.7	520.0	36.8	18.82
<i>Agaricus coniferarum</i>	C	152.0 ± 5.0	38.0 ± 4.1	164.0±3.8	54.5 ± 3.2	5.86±0.10
	D	188.0 ± 4.0	55.5 ± 5.0	263.0±5.2	10.7 ± 2.5	6.97±0.43
	M	387.0 ± 4.6	155.0 ± 2.9	428.0±5.0	30.7 ± 4.2	8.67±0.13
	Total	727.0	248.5	828.0	95.9	21.50
<i>Leucoagaricus litoralis</i>	C	71.1 ± 4.4	51.8 ± 4.8	75.0 ± 6.9	51.4 ± 3.5	5.12±0.23
	D	121.0 ± 4.2	70.5 ± 4.1	45.7 ± 4.6	70.8 ± 4.4	6.40±0.19
	M	239.0 ± 2.3	73.0 ± 5.0	175.0 ± 6.9	76.3 ± 4.8	6.09±0.44

Botanical name	Extract	$\mu\text{M TE/g edw}$				TPC mgGAE/gedw
		ABTS ⁺	DPPH ⁺	FRAP	ORAC	
	Total	431.1	195.3	295.7	198.5	17.61
<i>Leucoagaricus subolivaceus</i>	C	29.2 ± 4.2	32.6 ± 2.9	51.0 ± 4.6	32.8 ± 1.3	5.65±0.31
	D	97.6 ± 2.6	10.5 ± 2.9	109.0 ± 2.3	33.5 ± 3.5	5.97±0.53
	M	113.0 ± 4.3	28.0 ± 4.8	169.0 ± 4.0	5.6 ± 3.2	4.41±0.19
	Total	239.8	71.1	329.0	71.9	16.03
<i>Sericeomyces subvolvatus</i>	C	11.1 ± 1.9	29.3 ± 2.5	66.0 ± 2.0	92.5 ± 4.4	5.52±0.24
	D	109.0 ± 1.0	30.5 ± 4.8	56.0 ± 3.8	86.4 ± 4.3	6.88±0.06
	M	44.1 ± 1.9	73.0 ± 4.1	175.0 ± 4.6	106.0 ± 3.0	5.01±0.21
	Total	164.2	132.8	297.0	284.9	17.41
<i>Gyroporus ammophilus</i>	C	14.6 ± 2.6	80.5 ± 5.0	160.0 ± 2.3	109.0 ± 2.4	5.37±0.12
	D	45.1 ± 2.6	75.5 ± 2.9	130.0 ± 6.1	111.0 ± 1.2	9.64±0.25
	M	281.0 ± 4.4	137.0 ± 4.8	134.0 ± 4.6	119.0 ± 2.3	8.71±1.12
	Total	340.7	293.0	424.0	339.0	23.72
<i>Leccinum (Boletus) lepidus</i>	C	49.1 ± 2.5	23.0 ± 5.0	189.0 ± 5.2	102.0 ± 2.2	7.17±0.19
	D	93.1 ± 4.4	29.3 ± 2.5	148.0 ± 5.0	115.0 ± 2.4	11.28±0.34
	M	203.0 ± 3.7	96.8 ± 4.8	249.0 ± 4.0	146.0 ± 3.2	7.57±0.34
	Total	345.2	149.1	586.0	363.0	26.02
<i>Boletus impolitus</i>	C	7.6 ± 2.6	33.0 ± 5.0	63.0 ± 3.3	95.4 ± 2.2	6.53±0.27
	D	279.0 ± 1.6	26.3 ± 2.9	159.0 ± 4.6	118.0 ± 1.0	6.42±0.08
	M	258.0 ± 3.4	79.3 ± 4.8	382.0 ± 3.3	118.0 ± 1.7	5.49±0.30
	Total	562.6	138.6	604.0	331.4	18.44
<i>Boletus luridus</i>	C	108.0 ± 4.4	24.7 ± 2.9	270.0 ± 3.8	106.0 ± 2.1	5.20±0.09
	D	121.0 ± 4.2	24.3 ± 4.8	307.0 ± 4.6	116.0 ± 3.1	5.37±0.01
	M	247.0 ± 2.3	134.0 ± 2.5	271.0 ± 5.7	124.0 ± 1.2	6.32±0.24
	Total	476.0	182.92	848.0	346.0	16.89
<i>Boletus lupines</i>	C	23.1 ± 3.0	33.0 ± 2.5	207.0 ± 3.3	204.0 ± 2.1	6.29±0.12
	D	10.1 ± 4.4	69.3 ± 4.8	188.0 ± 6.0	74.3 ± 3.7	6.20±0.16
	M	210.0 ± 3.0	178.0 ± 4.1	277.0 ± 4.0	60.2 ± 4.4	8.13±0.19
	Total	343.2	280.3	672.0	338.5	20.62

Botanical name	Extract	$\mu\text{M TE/g edw}$				TPC mgGAE/gedw
		ABTS ⁺	DPPH ⁺	FRAP	ORAC	
<i>Xerocomus chrysenteron</i>	C	88.5 ± 2.5	5.00 ± 0.5	81.2 ± 1.4	26.5 ± 1.4	6.44 ± 0.31
	D	35.4 ± 1.7	32.6 ± 0.6	75.2 ± 4.9	16.4 ± 0.1	5.74 ± 0.14
	M	138.0 ± 4.5	79.5 ± 5.0	36.4 ± 3.5	49.3 ± 3.2	4.96 ± 0.18
	W	145.0 ± 3.1	98.5 ± 4.2	299.0 ± 4.0	11.9 ± 0.4	6.56 ± 0.12
	Total	406.9	215.6	491.8	104.1	23.70
<i>Stropharia halophila</i>	C	128.0 ± 2.6	35.5 ± 4.8	186.0 ± 3.8	6.7 ± 1.6	5.01 ± 0.15
	D	51.3 ± 3.1	30.5 ± 4.1	51.0 ± 4.6	9.0 ± 1.8	6.33 ± 0.20
	M	258.0 ± 4.8	34.3 ± 4.8	331.0 ± 3.3	20.3 ± 3.1	6.01 ± 0.49
	Total	432.3	100.3	568.0	36.0	17.35
<i>Tricholoma focale</i>	C	17.1 ± 4.4	40.5 ± 5.0	63.0 ± 4.0	23.3 ± 4.7	5.69 ± 0.24
	D	36.6 ± 3.7	34.3 ± 4.8	52.3 ± 2.3	58.7 ± 2.2	4.98 ± 0.16
	M	83.1 ± 4.4	39.3 ± 5.0	71.0 ± 4.0	55.1 ± 4.5	5.42 ± 0.07
	Total	136.8	114.1	186.3	137.1	16.09
<i>Tricholoma auratum</i>	C	74.1 ± 3.5	25.5 ± 5.0	46.0 ± 3.8	50.5 ± 2.9	7.26 ± 0.25
	D	207.0 ± 3.5	35.5 ± 4.1	67.0 ± 3.3	61.2 ± 1.2	10.70 ± 0.44
	M	299.0 ± 4.3	37.4 ± 3.8	254.0 ± 4.6	27.8 ± 2.3	6.07 ± 0.62
	Total	580.1	100.4	367.0	139.5	24.03
<i>Tricholoma caligatum</i>	C	31.6 ± 1.9	28.0 ± 5.0	287.0 ± 2.3	45.6 ± 4.9	9.60 ± 0.86
	D	95.6 ± 4.4	36.8 ± 2.5	332.0 ± 4.6	65.3 ± 1.2	11.41 ± 0.81
	M	91.1 ± 3.4	31.8 ± 4.8	207.0 ± 6.1	41.2 ± 4.4	9.65 ± 0.88
	W	35.9 ± 1.6	25.8 ± 4.1	173.0 ± 3.8	5.8 ± 1.0	5.89 ± 0.24
	Total	254.2	122.4	999.0	158.9	36.64
<i>Tricholoma columbetta</i>	C	35.0 ± 0.5	6.9 ± 1.0	120.2 ± 3.5	155.0 ± 1.0	6.25 ± 0.20
	D	33.1 ± 1.9	15.9 ± 1.3	65.2 ± 6.2	135.0 ± 3.0	4.74 ± 0.06
	M	47.9 ± 1.1	14.5 ± 3.1	9.1 ± 2.8	64.6 ± 3.6	6.60 ± 3.50
	W	160.0 ± 1.6	7.1 ± 2.3	10.7 ± 0.2	10.8 ± 0.7	5.18 ± 0.28
	Total	276.0	44.4	205.2	365.4	22.77
<i>Melanoleuca cinereifolia</i>	C	4.6 ± 1.6	21.3 ± 2.9	23.0 ± 3.3	17.2 ± 2.1	6.04 ± 0.29
	D	49.1 ± 2.5	20.5 ± 5.0	22.0 ± 3.9	109.0 ± 4.9	5.27 ± 0.16
	M	74.1 ± 5.0	66.8 ± 4.8	83.0 ± 4.0	100.0 ± 1.4	4.50 ± 0.24

Botanical name	Extract	$\mu\text{M TE/g edw}$				TPC mgGAE/gedw
		ABTS ⁺	DPPH ⁺	FRAP	ORAC	
	Total	127.8	108.6	128.0	226.2	15.81
<i>Clitocybe odora</i>	C	8.1 ± 1.6	31.8 ± 4.7	172.0 ± 3.8	95.5 ± 2.3	9.46 ± 0.95
	D	21.1 ± 2.6	28.0 ± 2.8	184.0 ± 2.3	55.3 ± 3.9	9.76 ± 0.43
	M	251.0 ± 4.4	177.0 ± 4.8	204.0 ± 4.6	41.6 ± 2.1	11.28 ± 0.86
	Total	280.2	236.8	560.0	192.4	30.50
<i>Amanita ovoidea</i>	C	8.5 ± 1.9	36.8 ± 4.8	24.0 ± 2.0	29.0 ± 3.4	7.02 ± 0.20
	D	23.1 ± 4.8	35.5 ± 4.1	40.0 ± 6.0	21.1 ± 2.1	7.95 ± 0.16
	M	41.1 ± 2.6	40.5 ± 4.1	93.0 ± 6.9	50.6 ± 3.7	5.21 ± 0.05
	Total	72.7	112.8	157.0	100.7	20.18
<i>Cortinarius suberretorum</i>	C	25.6 ± 1.9	41.8 ± 4.8	61.0 ± 4.0	73.4 ± 3.3	7.67 ± 0.17
	D	105.0 ± 2.6	154.3 ± 4.1	160.0 ± 3.3	82.2 ± 4.3	11.51 ± 0.52
	M	225.0 ± 4.8	45.5 ± 2.3	240.0 ± 3.8	101.0 ± 4.8	6.25 ± 0.17
	Total	355.6	241.6	461.0	256.6	25.43
<i>Cortinarius infractus</i>	C	63.6 ± 4.43	34.3 ± 4.8	236.0 ± 2.3	114.0 ± 3.6	8.40 ± 0.38
	D	102.0 ± 3.65	99.3 ± 2.5	304.0 ± 4.6	156.0 ± 1.8	12.06 ± 1.18
	M	483.0 ± 3.83	354.0 ± 4.8	569.0 ± 4.0	126.0 ± 3.5	17.72 ± 0.90
	Total	648.6	487.6	905.0	396.0	38.18
<i>Russula badia</i>	C	14.1 ± 1.6	24.3 ± 2.5	13.0 ± 2.3	15.8 ± 2.8	5.80 ± 0.19
	D	36.6 ± 3.4	21.8 ± 4.7	54.0 ± 5.0	23.2 ± 3.2	4.54 ± 0.18
	M	82.1 ± 4.3	45.5 ± 4.1	60.3 ± 4.6	23.8 ± 4.4	6.00 ± 0.53
	Total	132.8	91.6	127.3	62.8	16.34
<i>Lactarius vinosus</i>	C	7.6 ± 1.9	48.0 ± 4.1	108.0 ± 6.0	37.2 ± 2.5	10.16 ± 0.28
	D	4.1 ± 2.6	99.3 ± 4.7	77.0 ± 2.3	81.1 ± 2.6	9.36 ± 0.33
	M	17.6 ± 1.9	43.8 ± 2.3	207.0 ± 6.1	38.9 ± 3.2	5.08 ± 0.08
	Total	29.3	191.1	392.0	157.2	24.6
<i>Phaeolus schweinitzii</i>	C	48.2 ± 1.4	494.0 ± 4.0	11.9 ± 0.7	33.6 ± 1.9	7.07 ± 0.49
	D	417.0 ± 2.9	176.0 ± 3.6	110.0 ± 2.3	42.7 ± 2.5	9.38 ± 0.41
	M	1629.0 ± 3.5	1282.0 ± 3.5	874.0 ± 2.0	340.0 ± 3.0	31.88 ± 1.67
	W	794 ± 3.00	295.0 ± 5.0	970.0 ± 4.2	45.6 ± 2.7	8.20 ± 0.33
	Total	2888.2	2247.0	1965.9	461.9	56.53

Botanical name	Extract	$\mu\text{M TE/g edw}$				TPC mgGAE/gedw
		ABTS ⁺	DPPH ⁺	FRAP	ORAC	
<i>Inonotus hispidus</i>	C	9.9 ± 0.9	118.0 ± 4.2	109.0 ± 4.9	7.5 ± 0.1	4.79 ± 0.36
	D	587.0 ± 1.4	225.0 ± 3.1	246.0 ± 6.4	18.6 ± 3.2	9.70 ± 0.64
	M	861.0 ± 5.0	1265.0 ± 5.0	883.0 ± 9.1	290.0 ± 1.0	28.91 ± 1.49
	W	1651.0 ± 2.5	1160.0 ± 5.0	3010.0 ± 4.5	53.1 ± 1.1	41.27 ± 0.86
	Total	3108.9	2768.0	4248.0	369.2	84.47
<i>Hydnellum ferrugineum</i>	C	16.7 ± 4.1	7.3 ± 2.1	170.0 ± 4.9	38.4 ± 1.9	7.22 ± 0.53
	D	108.0 ± 5.0	40.2 ± 4.5	180.0 ± 4.2	90.7 ± 1.2	6.92 ± 0.41
	M	138.0 ± 3.8	79.5 ± 5.0	117.0 ± 9.9	74.0 ± 3.1	8.05 ± 0.23
	W	1074.0 ± 2.5	215.0 ± 5.0	1230.0 ± 0.7	63.5 ± 3.3	13.31 ± 0.07
	Total	1336.7	342.0	1697.0	266.6	35.50

Results are expressed as a mean ± standard deviation (n=4); C, cyclohexane; D, dichloromethane; M, methanol; W: water.

3.1.2. Antioxidant capacity differences between mushroom species

The highest antioxidant potential demonstrated *I. hispidus* and *P. schweinitzii* extracts, particularly in SET assays, while the extracts isolated from such species as *L. vinosus* and *T. columbetta* were the weakest antioxidant sources in these assays. For instance, the sum of TEAC of *I. hispidus* extracts in ABTS⁺ scavenging assay (3108.9 $\mu\text{M TE/g}$) was more than 100 times higher comparing to *L. vinosus* (29.3 $\mu\text{M TE/g}$). These differences were less remarkable in other SET assays, while the ORAC values varied from 36.0 (*S. halophila*) to 461.9 $\mu\text{M TE/g}$ (*P. schweinitzii*). TPC was from 15.17 (*A. devoniensis*) to 84.47 mg GAE/g (*I. hispidus*). However, extract yields were dependent both on mushroom species and extraction solvent, therefore TPC values obtained for extracts were recalculated for 1 g of mushroom dry weight (mdw), taking into account how much of TPC is extracted with each solvent. The TPC values expressed in this way are presented in Fig. 3.2: they were from 0.51 mg GAE/g mdw (*S. halophila*) to 5.90 mg GAE/g mdw (*I. hispidus*). In general the TPC values in most cases were in agreement with antioxidant capacity values obtained in other assays.

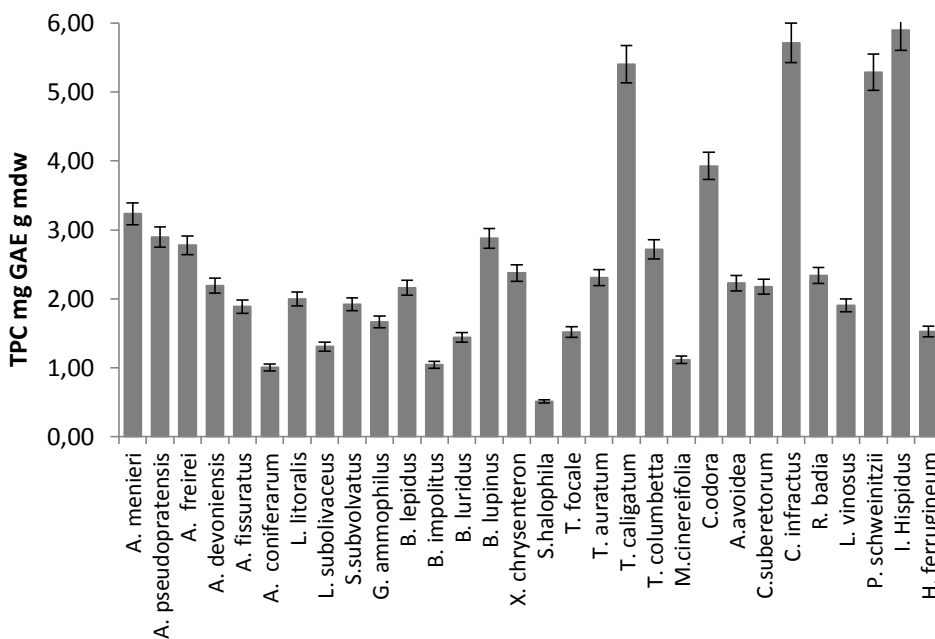


Fig. 3.2 Total content of phenolic compounds (TPC) in mushrooms

Taking into account all measured characteristics, the ASMs of wild mushrooms expressed in $\mu\text{g}/\text{g mdw}$ may be grouped in four classes (Fig. 3.3) with antioxidant scores 0-100 (8 species), 100-200 (12 species), 200-300 (6 species) and more than 300 $\mu\text{g}/\text{g mdw}$ (4 species). Several species (*I. hispidus*, *P. schweinitzii*, *C. infractus*) were of superior antioxidant potential comparing with the majority of

studied mushroom species; their integrated antioxidant scores were in the range of 481-808 $\mu\text{g}/\text{g}$ mdw; thus Agaricaceae (109-325 $\mu\text{g}/\text{g}$ mdw) and Boletaceae (140-253 $\mu\text{g}/\text{g}$ mdw) families species were with higher integrated antioxidant score than Tricholomataceae (47-206 $\mu\text{g}/\text{g}$ mdw) and the rest of tested families.

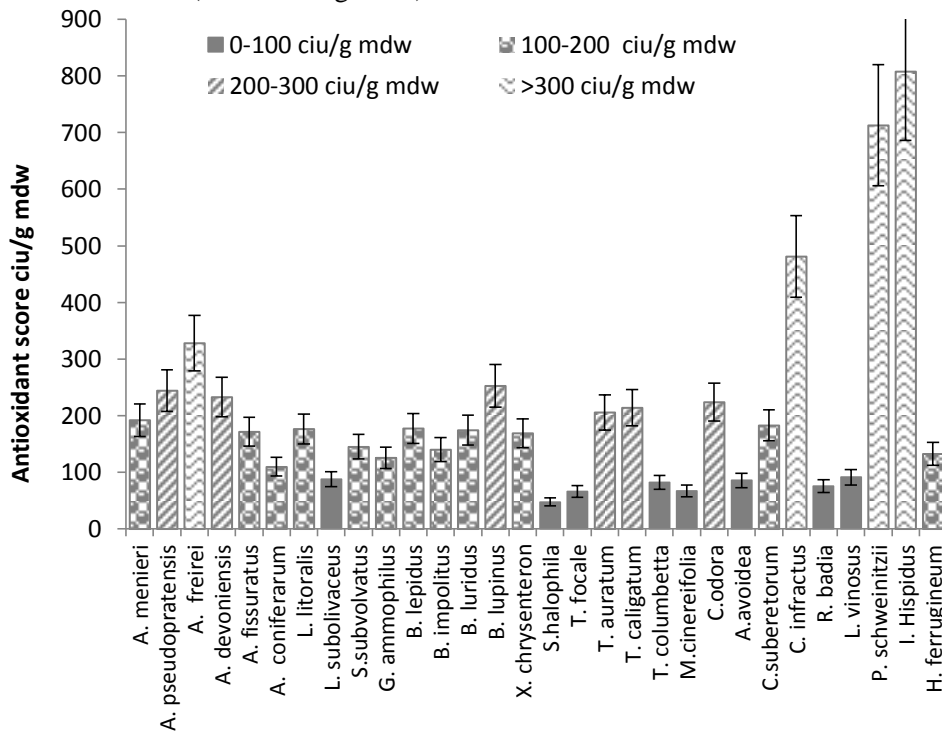


Fig. 3.3 Antioxidant scores of mushrooms species integrating antioxidant activity values

The RSC of *A. devoniensis* methanol extract was reported to increase with higher concentration (Al-Fatimi et al., 2005); ethanol and water soluble polysaccharide fraction of *C. odora* was weaker DPPH \cdot and OH \cdot scavenger and possessed lower reducing properties comparing with other 4 tested commercial mushroom species (Vaz et al., 2011); however, in the mentioned studies antioxidant indicators were expressed in percentage of scavenged radicals and therefore are difficult to compare with our results. More effective mushrooms species were reported to contain higher amounts of secondary metabolites such as phenolics exerting multiple biological effects including antioxidant activity (Kim et al., 2008).

3.1.3. Antioxidant capacity differences between extractant

Generally polar solvents are most regularly used for the extraction of antioxidants from materials containing polyphenolics as the main antioxidatively active compounds; however, some fungi origin materials may also contain lipophilic compounds such as tocopherols, carotenoids, terpenoids and the use of different polarity solvents may provide more comprehensive information on their antioxidant potential, particularly in case of less studied mushroom species. Our results clearly

demonstrate (Table 3.3) that distribution of antioxidatively active constituents in the fractions isolated with different solvents is highly dependent on mushroom species. The extracts isolated from *I. hispidus* and *P. schweinitzii* (the species possessing the highest antioxidant potential) with protic solvents methanol and water were remarkably stronger antioxidants than cyclohexane and dichloromethane extracts of the same species in all assays, while for other species the results are more complicated. For instance, cyclohexane extracts of 7 mushrooms species were 10-30% stronger antioxidants in SET and/or HAT assays than dichloromethane (*A. freirei*, *A. devoniensis*, *A. fissuratus*, *B. lupinus*, *S. halophila*, *T. focale*, *C. odora*), while other 20 species results were similar and activity increased with protic solvent. *T. columbetta* or *X. chrysenteron* (except DPPH[•] assay) cyclohexane fractions in all tested assays were from 10-60% higher than dichloromethane fractions. However, it should be mentioned that these species were characterized as sufficient antioxidant potential. It is interesting noting that dichloromethane fractions of species *A. menieri*, *A. pseudopratisensis*, *B. luridus*, *T. caligatum*, possessed stronger, sometimes 40% higher antioxidant activity than methanol fraction in FRAP, while the TPC values were respectively higher for these fractions. *A. menieri*, *A. freirei*, *L. subolivaceus*, *B. lupinus*, *T. auratum*, *L. vinosus* values in ORAC assay were 10-50% higher than methanol and indicated dichloromethane as appropriate solvent for extracting phenols.

Our study shows that methanol and water may be useful solvent for the extraction of antioxidants from some mushroom species after applying polar organic solvents. Water is a preferable solvent in terms of toxicity and availability; however, it is not always sufficiently efficient for the isolation for mushroom bioactive compounds. For instance, water extract of *I. hispidus* was strongest antioxidant in ABTS^{•+}, FRAP and TPC assays; however, it was almost 6 times weaker in ORAC assay compared to methanol fraction. It is also important noting that the yield of water extract from *I. hispidus* was remarkably higher than the yields obtained with other solvents; water fraction constituted 67% of the total extractives. Water extracts of other 6 tested species were also remarkably less effective in ORAC assay, except for *H. ferrugineum*, when the difference between methanol and water fractions constituted only 14%. It should be noted that water extracts were obtained by boiling the residues of extractions with organic solvents and in this case some hydrolysis and other processes involving chemical changes may occur in extraction material. Generally, methanol extracts of most species were strongest in ABTS^{•+}, DPPH[•] scavenging, FRAP and the yields were remarkably higher than the yields obtained with other solvents; however cyclohexane and dichloromethane fractions were several times weaker radical scavengers than polar methanol extracts, except several species described above. Many previously performed studies reported that polar solvents extract more antioxidants from botanicals than lower polarity solvents (Brahmi et al., 2012), which is not true for some wild mushrooms species. Integrated antioxidant potential scores were also calculated for the extracts isolated with different solvents for comparative assessment of the effectiveness of each solvent for the tested wild mushroom species Figure 3.4 and according to the score levels (ciu/g edw) the cyclohexane, dichloromethane and methanol extracts were classified

into the 5 groups and expressed in percentage (Figure 3.5). However, it should be noted that in this case the scores were calculated by summing the values measured for 1 g of extracts dw (Fig. 3.4) are not associated with the antioxidant scores calculated in $\mu\text{g}/\text{g}$ mdw, which are presented in Fig. 3.3.

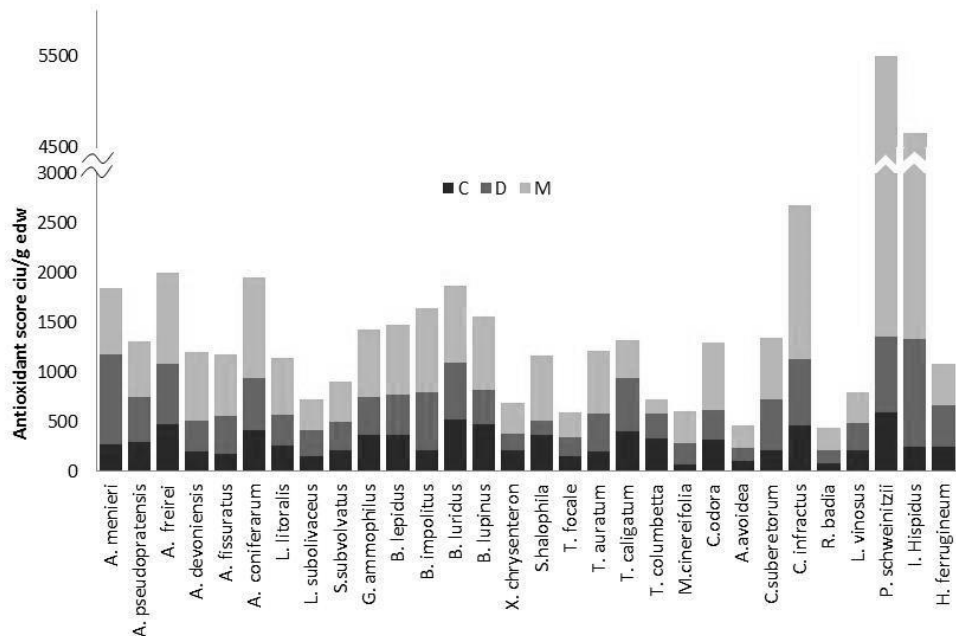


Fig 3.4 Antioxidant scores of mushrooms extracts integrating antioxidant activity values

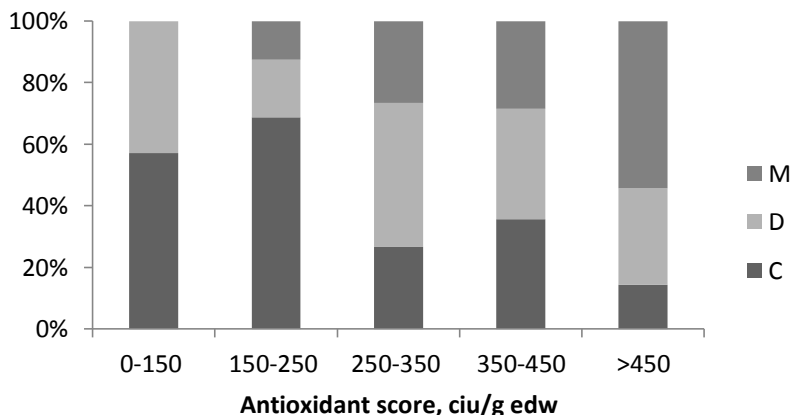


Fig. 3.5 Antioxidant scores of extracts (ASE) according to score levels and solvent polarity expressed in percentage.

3.1.3. Antioxidant capacity differences between extraction method

Antioxidant activity values obtained by using different evaluation assays are in a very wide range, they depend both on mushroom species and extracted fraction. Generally the highest TEAC values were obtained in FRAP assay; their sum from all fractions were from 128.0 (*M. cinereifolia*) to 4248.0 $\mu\text{M TE/g}$ (*I. hispidus*). ABTS^{++} values were from 29.3 (*L. vinosus*) to 3108.9 $\mu\text{M TE/g}$ (*I. hispidus*); DPPH^{\bullet} scavenging assay values were from 44.4 (*T. columbetta*) to 2768.0 $\mu\text{M TE/g}$ (*I. hispidus*), while the lowest values were measured in ORAC, from 36.0 (*S. halophila*) to 461.9 $\mu\text{M TE/g}$ (*P. schweinitzii*). Strong correlation was observed between ABTS^{++} and FRAP ($R^2 = 0.9095$) as well as DPPH^{\bullet} and ABTS^{++} ($R^2 = 0.9498$); Also total phenols had strong correlation with DPPH^{\bullet} ($R^2 = 0.9043$), FRAP ($R^2 = 0.9230$) and ABTS^{++} ($R^2 = 0.8842$), confirming that phenolic compounds are important contributors to the antioxidant properties of these extracts. However, the correlation between TPC and ORAC was weaker ($R^2 = 0.44$).

Several reasons may be considered to explain the obtained differences between the applied assays. Although the principle of the applied radical scavenging or reduction assays are based on SET and/or HAT, the peculiarities of reaction mechanisms in each assay are different; they may largely depend on reaction media, pH, the structure of antioxidative compounds present in the extracts, their interactions and other factors. For instance, Agaricaceae, Boletaceae and Cortinariaceae families species possessed high antioxidant capacities in ABTS^{++} and values were from 164.2 (*S. subvolvatus*) to 665.6 (*A. menieri*) $\mu\text{M TE/g}$, comparing to lower Tricholomataceae family species (127.8-580.1 $\mu\text{M TE/g}$); remaining families demonstrated weaker activity, except three forest mushroom species *H. ferrugineum* (1336.7 $\mu\text{M TE/g}$), *P. schweinitzii* (2888.2 $\mu\text{M TE/g}$), *I. hispidus* (3108.9 $\mu\text{M TE/g}$) with highest antioxidant capacity. As Zan et al. (2011), reported that in ABTS^{++} scavenging assay, 5 compounds isolated from *I. hispidus* methanol extract exhibited significant activity, from 12.71 ± 3.57 to 59 ± 9.70 $\mu\text{M TE}/\mu\text{M}$ compound. These findings support our results indicating high ABTS^{++} scavenging capacity of *I. hispidus* water and methanol fractions. For example, in reported studies five edible wild Australian mushrooms, *Morchella elata* (240 ± 22 $\mu\text{M TE/g}$), *Flammulina velutipes* (221 ± 20 $\mu\text{M TE/g}$), *Suillus luteus* (154 ± 14 $\mu\text{M TE/g}$), *Pleurotus eryngii* (67.0 ± 0.6 $\mu\text{M TE/g}$) and *Cyttaria gunnii* (77.0 ± 0.7 $\mu\text{M TE/g}$) were evaluated for their antioxidant capacity and methanol fractions were reported as strong ABTS^{++} scavengers (Zeng et al., 2012). The values obtained in our study for some species were similar, while for other species were two or three times higher than those measured for Australian edible mushrooms.

The sum of values measured in DPPH^{\bullet} scavenging assay of Boletaceae and Cortinariaceae families species demonstrated high antioxidant capacity and values were 138.6-487.6 $\mu\text{M TE/g}$ (*B. impolitus* and *C. infractus* respectively), comparing to not significantly lower Agaricaceae (71.1-264.0 $\mu\text{M TE/g}$) and Tricholomataceae (44.4-236.8 $\mu\text{M TE/g}$) families; remaining families demonstrated similar DPPH^{\bullet} scavenging values, except *P. schweinitzii* (2247.0 $\mu\text{M TE/g}$), *I. hispidus* (2768.0 $\mu\text{M TE/g}$) with highest antioxidatively active components. Antioxidant properties of *A.*

devonienis extracts sequentially isolated by different polarity solvents previously, (dichloromethane methanol and water) while methanol fraction depending on extract concentration, inhibited from 3.3-65.5%; the results were comparable with the activity of the reference compound ascorbic acid 48.8-97.2% (Al-Fatimi et al., 2005), i.e. the units which are not applicable for comparison purposes. DPPH[•] scavenging capacity was also recently reported for *B. impolitus* and evaluated by measuring their effective methanol value $EC_{50}=5.81 \pm 0.17$ mg/mL (Pereira et al., 2012); thus are difficult to compare with our results obtained for *B. impolitus* methanolic fraction (79.3 μ M TE/g). Effective DPPH[•] scavenging concentration EC_{50} of *C. odora* ethanol and water soluble polysaccharide extracts evaluated previously; ethanol fraction was stronger antioxidant comparing to water soluble polysaccharide extracts in DPPH[•] assay, then in reducing power and β -carotene bleaching inhibition assays (Vaz et al., 2011, Suay et al., 2000). Effective DPPH[•] scavenging concentration EC_{50} of *X. chrysenteron* methanol/water extract was 2.06 ± 0.46 mg/mL (Heleno et al., 2012), while methanol fraction, depending on extract concentration, inhibited from 27.42 ± 1.23 to 89.61 ± 0.10 % DPPH[•] (Sarikurkcu et al., 2008). Although we used different extraction procedure and antioxidant activity assays, some agreement in the obtained results may be observed, particularly in case of DPPH[•] scavenging assay, when in both studies more polar solvent applied more stronger antioxidants values in DPPH[•] assay found. However some species have the exception and twice effectively antioxidant capacity shows dichloromethane fractions, such as species *A. menieri*, *L. vinosus*.

The third method used to evaluate antioxidant potential of mushroom species was FRAP. In case of this assay highest total extracts efficiency belongs to forest mushrooms *H. ferrugineum* (1697.0 μ M TE/g), *P. schweinitzii* (1965.9 μ M TE/g), *I. hispidus* (4248.0 μ M TE/g); Lower reducing ability had Cortinariaceae (461.0-905.0 μ M TE/g) Tricholomataceae (128.0-999.0 μ M TE/g) Boletaceae (491.8-848.0 μ M TE/g) and Agaricaceae (297.0-828.0 μ M TE/g) families; other species also possessed effective activity and varied from 127.3 (*R. badia*) to 568.0 μ M TE/g (*S. halophila*). FRAP was also used in some other studies of mushrooms; however, their data is difficult to compare due to different units used to express the data. Reducing power of *B. impolitus* (Pereira et al., 2012) *C. odora* (Vaz et al., 2011, Suay et al., 2000) and *X. chrysenteron* (Sarikurkcu et al., 2008) methanol extracts was reported previously as well, however, it was also expressed in relative units. However in FRAP assay as well as in DPPH[•] scavenging assay are several mushroom species with more powerful reducing ability in cyclohexane or dichloromethane fractions (*H. ferrugineum*, *T. columbetta*), than described in polar fraction. For example, *A. menieri* and *A. pseudopratisensis* dichloromethane fraction were 20-30% more efficient than methanol fraction, and these mushrooms are noticeable as species with strong phenolic odour. Chelating agents may act as secondary antioxidants by reducing redox potential and stabilizing the oxidised forms of metal ions (Mishra et al., 2013).

Finally, the antioxidant activity of mushrooms was evaluated using ORAC assay; the total values for investigated species with highest activity were forest mushrooms *P. schweinitzii* (461.9 μ M TE/g), *I. hispidus* (369.2 μ M TE/g) and dune

families Cortinariaceae (396.0-257.0 $\mu\text{M TE/g}$), Gyroporaceae (339.0 $\mu\text{M TE/g}$); all Boletaceae family species demonstrated strong antioxidant activity and ORAC values were 331.4-104.1 $\mu\text{M TE/g}$; remarkably strong peroxy radical inhibitors showed *B. lupinus* cyclohexane fraction 204.0 $\mu\text{M TE/g}$ whereas dichloromethane and methanol was lower 74.3 - 60.2 $\mu\text{M TE/g}$ respectively. Other Boletaceae species possessed similar activity, where methanol extracts were dominated in ORAC assay. Some Agaricaceae family species demonstrated also high activity, such as *A. freirei* (394.0 $\mu\text{M TE/g}$), *A. pseudopratisensis* (305.8 $\mu\text{M TE/g}$), while other species the same family were with ten to five times lower activity (*A. fissuratus* 36.8 $\mu\text{M TE/g}$, *L. subolivaceus* 71.9 $\mu\text{M TE/g}$). Tricholomataceae family species possessed twice lower activity for tested active species in ORAC assay 137.1 (*T. focale*) – 226.2 (*M. cinereifolia*) $\mu\text{M TE/g}$. Amanitaceae, Strophariaceae, and Russulaceae families were with fifty of twenty times lower than most active mushrooms species in this assay. As far as we know, ORAC activity of tested mushrooms species has been characterized for the first time.

The values measured with Folin–Ciocalteu reagent and expressed in gallic acid or other phenolic compound are generally accepted as representing the total phenolic content (TPC) although it is not fully correct: Folin–Ciocalteu reagent reacts not only with phenolics but with other reducing ability possessing compounds in the reaction system (Huang et al., 2005). Consequently the term TPC may be used rather conditionally; however, for the convenience we are using this term in our study. Thus, the integrated TPC values (their sum in the all extracts) highest phenolic content possessed *P. schweinitzii* (56.53 mg GAE/g) and *I. hispidus* (84.47 mg GAE/g), following by *C. infractus* 38.2 mg GAE/g edw and *H. ferrugineum* 35.5 mg GAE/g edw, Tricholomataceae family distributed values 15.8-36.5 mg GAE/g edw, it is interesting to noting that dichloromethane fractions of *T. auratum* and *T. caligatum* were aprx.40% and 20% respectively higher than methanol or cyclohexane fractions. Agaricaceae family species also demonstrated high TPC value 15.2-35.3 mg GAE/g edw, for instance, *A. menieri*, possessed highest TPC value of dichloromethane fraction (14.29 GAE/g edw) in this assay. Other species of this family also indicated higher phenolic content in dichloromethane fractions, except *A. coniferarum* and *A. devoniensis*, were major TPC values possessed polar protic solvent M. Boletaceae family species TPC sum values were similar and warried from 16.9 (*B. luridus*)– 26.0 GAE/g edw (*B. lepidus*). For instance, the methanol fraction of *B. impolitus* (15.50 \pm 0.53 GAE/g edw) (Pereira et al., 2012) and water/ethanol (9:1) fraction of *C. odora* (5.69 GAE/g edw) (Del Signore, Romeo and Giaccio, 1997). extracts is lower than the sum of TPC in all extracts obtained from this species in our study (Fig. 3.2), 18.4 and 30.5 GAE/g edw respectively; however, the extracts in previous study was obtained from the whole material, while in our study methanol was used for reextracting the residue after cyclohexane and dichloromethane extraction.

Antioxidant potential of studied mushrooms was found to be in a rather wide range: the differences were observed between the tested species as well as between the fractions isolated by different solvents. Assay method was also important factor in determining antioxidant properties of mushroom extracts. The extracts isolated

with methanol and water from *P. schweinitzii* and *I. hispidus* were most powerful antioxidants almost in all tested assays.

3.3. ANTIBACTERIAL AND BIOLOGICAL PROPERTIES OF COLLECTED WILD MUSHROOM SPECIES

Worldwide, bacterial infections have increased dramatically over the past few years (Theuretzbacher, 2013). Microorganisms and parasites are becoming more and more resistant. The most common and effective treatment of infectious disease till now has been the application of drugs with antimicrobial activity. Drug resistance usually occur after long-term misuse of antibacterial substances and bacterial survival is a consequence of the acquisition of mutations in the bacterial genome and genes. Nowadays it is impossible to avoid bacterial or parasites evolution, it is substantial choose the most appropriate antibiotic and to use them appropriately to minimize the growth of resistant microorganisms. There will forever be demand for novel substances or compounds to outstrip bacteria and other pathogens (Ren et al., 2014). Many species of fungus produce antibacterial and antifungal agents to survive in natural environment. These metabolites are the major sources of pharmacologically active drugs or food preservatives including antibiotics, alkaloids, phenols, gallic acids, free fatty acids and their derivatives (Lindequist et al., 2005, Gyawali and Ibrahim, 2014).

3.3.1. Antibacterial properties of mushroom extracts

Evaluation of antioxidant activity of extracts isolated from the collected mushroom species by different solvents revealed remarkable variability in the obtained values. It is known that many antioxidatively active compounds may also possess different effects against microorganisms.

An antimicrobial activity was considered to be an indicator of the mushrooms species to produce bioactive secondary metabolites of potential therapeutic interest. Therefore, the study was continued for the preliminary screening of antimicrobial properties of the extracts against two Gram-positive and two Gram-negative bacteria. The results obtained for 80 extracts isolated from 30 mushroom species are summarized in *Table 3.3* listing the inhibition zones in the agar diffusion assay at different applied concentrations. Appendix 1 gives an overview of the sorted families and the number of active species against Gram-positive and Gram-negative bacteria. It is apparent from *Table 3.3* that representatives from the different genera marked differences in their ability to show antimicrobial activities. Species of the *B. impolitus*, *B. luridus*, *G. ammophilus*, *S. halophila*, *T. caligatum*, *C. infractus* were especially productive (75-95% activity) while members of family Agaricaceae, species of the *B. lepidus*, *B. lupinus*, *T. focale*, *T. auratum*, *C. odora*, *A. ovoidea*, *R. badia*, *L. vinosus*, *C. suberretorum*, *M. cinereifolia* gave intermediate results (30-50% activity). For instance, the largest inhibition zones more than 15mm was observed for methanol extract of *P. schweinitzii* and *I. hispidus* against *P. aeruginosa* and *B. cereus* respectively; Also strong effect (>15mm) demonstrated methanol extract of *C. infractus* with partial activity against *P. aeruginosa*. It is interesting noting that *I. hispidus*, *P. schweinitzii* and *C. infractus* possessed the highest antioxidant potential,

comparing with other studied species, which were less active in antimicrobial tests. The differences between the representatives of all of the species might reflect the diverse substances of these mushrooms and have couple of effects; it may have strong activity, inhibit all microorganism growth or have partially inhibition activity around the disc. The test mushroom species showed greater antibacterial activity against Gram-positive than Gram-negative bacteria. For instance, tested bacteria were not inhibited 11% of the tested extracts (*Appendix 1*). Comparing the solvents, methanol extracts in most cases were the strongest antimicrobial agents, whereas *Agaricaceae* family dichloromethane fraction possessed the strongest inhibitory activity except *A. devoniensis* and *L. subolivaceus* extracts which was not tested. Methanol and cyclohexane fraction of *Boletaceae* family had very similar activity, except species of *B. lupinus* methanol extract showed stronger activity.

Table 3.3 Antimicrobial activity of mushroom extracts with inhibition zones

Botanical name	fraction	<i>Staphylococcus aureus</i>			<i>Bacillus cereus</i>			<i>Pseudomonas aeruginosa</i>			<i>Escherichia coli</i>		
		20 mg/mL	10 mg/mL	5 mg/mL	20 mg/mL	10 mg/mL	5 mg/mL	20 mg/mL	10 mg/mL	5 mg/mL	20 mg/mL	10 mg/mL	5 mg/mL
<i>Agaricus menieri</i>	D	(++)	(+)	(+)	(++)	(+)	-	++	++	+	(++)	(+)	(+)
	M	(++)	(+)	-	-	-	-	-	-	-	+	-	-
<i>Agaricus pseudoprattensis</i>	C	(++)	(++)	(+)	(+)	(+)	(+)	(++)	-	-	-	-	-
	D	(++)	(+)	-	(++)	(+)	-	++	+	+	(++)	(+)	(+)
	M	+	-	-	+	+	-	+	-	-	(+++)	(+)	-
<i>Agaricus freirei</i>	C	-	-	-	(+)	(+)	(+)	(+)	(+)	-	-	-	-
	D	(+)	(+)	(+)	-	-	-	(++)	(++)	(+)	-	-	-
	M	(+)	(+)	-	-	-	-	-	-	-	-	-	-
<i>Agaricus devoniensis</i>	M	+	-	-	-	-	-	+	+	-	-	-	-
<i>Agaricus fissuratus</i>	D	++	(++)	(++)	(++)	(+)	(+)	-	-	-	(++)	(++)	(+)
	M	+	-	-	+	+	-	+	+	-	-	-	-
<i>Agaricus coniferarum</i>	D	(+)	(+)	-	(+)	(+)	-	(+)	(+)	(+)	(+)	(+)	(+)
	M	-	-	-	(++)	(+)	-	-	-	-	+	-	-
<i>Leucoagaricus litoralis</i>	D	+	+	-	-	-	-	-	-	-	(++)	(++)	(+)
	M	+	+	-	(++)	(+)	-	-	-	-	+	+	-
<i>Leucoagaricus subolivaceus</i>	M	-	-	-	+	-	-	+	+	-	-	-	-
<i>Sericeomyces subvolvatus</i>	C	(++)	(+)	(+)	(+)	(+)	(+)	-	-	-	-	-	-
	D	++	+	+	(++)	(+)	(+)	-	-	-	-	-	-
	M	-	-	-	+(++)	+(++)	(+)	+	+	-	-	-	-

Botanical name	fraction	<i>Staphylococcus aureus</i>			<i>Bacillus cereus</i>			<i>Pseudomonas aeruginosa</i>			<i>Escherichia coli</i>		
		20 mg/mL	10 mg/mL	5 mg/mL	20 mg/mL	10 mg/mL	5 mg/mL	20 mg/mL	10 mg/mL	5 mg/mL	20 mg/mL	10 mg/mL	5 mg/mL
<i>Gyroporus ammophilus</i>	C	(++)	(+)	-	(+)	(+)	-	-	-	-	-	-	-
	D	(++)	(++)	-	(+++)	(++)	(+)	(++)	-	-	(++)	(++)	(++)
	M	+	+	+	(+++)	(++)	(+)	(++)	(++)	(+)	-	-	-
<i>Boletus lepidus</i>	C	(++)	(++)	(+)	(++)	(+)	(+)	-	-	-	(+)	(+)	-
	D	(+++)	(++)	(++)	-	-	-	(++)	(++)	(+)	-	-	-
	M	-	-	-	+	-	-	-	-	-	++	+	-
<i>Boletus impolitus</i>	C	++	+	(+)	(++)	(+)	(+)	(++)	(+)	(+)	-	-	-
	M	++(+++)	++(+)	-	(++)	(+)	(+)	++	+	+	(++)	(++)	-
<i>Boletus luridus</i>	C	+++	++	++	(++)	(+)	(+)	(++)	(+)	-	-	-	-
	M	+(+++)	++(+)	-	-	-	-	(+++)	(+++)	(+)	+	-	-
<i>Boletus lupinus</i>	C	-	-	-	-	-	-	(+)	(+)	(+)	-	-	-
	D	(++)	(++)	-	(++)	(+)	(+)	-	-	-	(++)	(+)	(+)
	M	+	+	+	-	-	-	(++)	(++)	(+)	-	-	-
<i>Xerocomus chryseron</i>	C	n.t	n.t	n.t	-	-	n.t	+	+	n.t	n.t	n.t	n.t
	D	n.t	n.t	n.t	-	-	n.t	+	+	n.t	n.t	n.t	n.t
	M	n.t	n.t	n.t	++	-	n.t	++	+	n.t	n.t	n.t	n.t
<i>Stropharia halophila</i>	C	-	-	-	-	-	-	-	-	-	(+)	(+)	-
	D	(+)	(+)	(+)	-	-	-	-	-	-	-	-	-
	M	+(+++)	++(+++)	(+)	+	-	-	(++)	(+)	-	-	-	-
<i>Tricholoma focale</i>	C	++	+	-	(+)	-	-	(++)	(+)	(+)	-	-	-
	D	(+)	-	-	-	-	-	-	-	-	(++)	(+)	(+)
	M	(+)	(+)	(+)	(++)	(++)	(+)	(++)	(++)	(+)	-	-	-
<i>Tricholoma</i>	C	-	-	-	-	-	-	-	-	-	(++)	(+)	(+)

Botanical name	fraction	<i>Staphylococcus aureus</i>			<i>Bacillus cereus</i>			<i>Pseudomonas aeruginosa</i>			<i>Escherichia coli</i>		
		20 mg/mL	10 mg/mL	5 mg/mL	20 mg/mL	10 mg/mL	5 mg/mL	20 mg/mL	10 mg/mL	5 mg/mL	20 mg/mL	10 mg/mL	5 mg/mL
<i>auratum</i>	D	(++)	(+)	(+)	-	-	-	-	-	-	-	-	-
	M	(++)	(+)	-	(++)	(+)	(+)	+	+	-	++	+	-
<i>Tricholoma caligatum</i>	C	(+)	(+)	(+)	-	-	-	-	-	-	-	-	-
	D	(++)	(++)	(+)	(++)	(++)	(++)	++	++	+	(++)	(+)	(+)
	M	+	+	+	(+++)	(++)	(++)	++	(++)	(+)	-	-	-
<i>Tricholoma columbetta</i>	C	n.t	n.t	n.t	+	-	n.t	+	+	n.t	n.t	n.t	n.t
	D	n.t	n.t	n.t	++	-	n.t	+	+	n.t	n.t	n.t	n.t
	M	n.t	n.t	n.t	++	++	n.t	++	+	n.t	n.t	n.t	n.t
<i>Melanoleuca cinereifolia</i>	C	(++)	(+)	(+)	-	-	-	-	-	-	(++)	(+)	(+)
	M	-	-	-	-	-	-	+	-	-	-	-	-
<i>Clitocyb odora</i>	D	(++)	(+)	(+)	-	-	-	(++)	(++)	(+)	-	-	-
	M	(+)	(+)	(+)	(++)	(+)	(+)	(++)	(+)	(+)	(++)	(+)	(+)
<i>Amanita ovoidea</i>	C	-	-	-	-	-	-	(++)	(+)	(+)	-	-	-
	D	-	-	-	-	-	-	++(+++)	(+)	(+)	(++)	(+)	(+)
	M	++	+	+	(++)	(+)	-	(++)	(+)	(+)	-	-	-
<i>Cortinarius suberretorum</i>	C	-	-	-	(++)	(+)	(+)	(+)	-	-	-	-	-
	M	-	-	-	-	-	-	-	-	-	(++)	(+)	-
<i>Cortinarius infractus</i>	D	++	(++)	(+)	(++)	(++)	(++)	++	+(++)	+(++)	-	-	-
	M	(+)	(+)	(+)	(++)	(++)	(++)	++(++++)	+(++)	(++)	(++)	(++)	(++)
<i>Russula badia</i>	D	-	-	-	-	-	-	-	-	-	-	-	-
	M	-	-	-	+(+++)	+(+++)	(+)	+	-	-	(++)	(+)	-
<i>Lactarius</i>	D	-	-	-	-	-	-	++	+	+	-	-	-

Botanical name	fraction	<i>Staphylococcus aureus</i>			<i>Bacillus cereus</i>			<i>Pseudomonas aeruginosa</i>			<i>Escherichia coli</i>		
		20 mg/mL	10 mg/mL	5 mg/mL	20 mg/mL	10 mg/mL	5 mg/mL	20 mg/mL	10 mg/mL	5 mg/mL	20 mg/mL	10 mg/mL	5 mg/mL
<i>vinosus</i>	M	+	+	+	-	-	-	(++)	(+)	(+)	(++)	(++)	(+)
<i>Phaeolus schweinitzii</i>	C	n.t	n.t	n.t	++	+	n.t	+	+	n.t	n.t	n.t	n.t
	D	n.t	n.t	n.t	+++	+	n.t	+	-	n.t	n.t	n.t	n.t
	M	n.t	n.t	n.t	+++	+++	n.t	+++	+++	n.t	n.t	n.t	n.t
<i>Inonotus hispidus</i>	C	n.t	n.t	n.t	+	+	n.t	+	+	n.t	n.t	n.t	n.t
	D	n.t	n.t	n.t	+	+	n.t	+	+	n.t	n.t	n.t	n.t
	M	n.t	n.t	n.t	++	+	n.t	+++	++	n.t	n.t	n.t	n.t
	W	n.t	n.t	n.t	-	-	n.t	++	+				
<i>Hydnellum ferrugineum</i>	C	n.t	n.t	n.t	-	-	n.t	-	-	n.t	n.t	n.t	n.t
	D	n.t	n.t	n.t	+	+	n.t	-	-	n.t	n.t	n.t	n.t
	M	n.t	n.t	n.t	-	-	n.t	+++	++	n.t	n.t	n.t	n.t
Control	C	-	-	-	-	-	-	-	-	-	-	-	-
	D	-	-	-	-	-	-	-	-	-	-	-	-
	M	-	-	-	-	-	-	-	-	-	-	-	-
	W	-	-	-	-	-	-	-	-	-	-	-	-

C, cyclohexane; D, dichloromethane; M, methanol; W: water; n.t: not tested; -: No inhibition zone. +: Inhibition zone 7-10 mm. ++: Inhibition zone 10-15mm. +++: Inhibition zone >15mm. (+): partial active zones 7-10mm. (++): partial inhibition zone 10-15mm. (+++): partial inhibition zone >15mm. Fractions which did not form any inhibition zones at all applied concentrations and the tested bacteria: *A. menieri* (C), *A. fissuratus* (C), *A. coniferarum* (C), *L. litoralis* (C), *B. impolitus* (D) *M. cinereifolia* (D), *C. infractus* (C), *R. badia* (C), *L.vinosus* (C); Fractions which did not tested in this assay: *A.devoniensis* (C,D), *L.subolivaceus* (C, D), *C. odora* (C), *B.luridus* (D), *C. infractus* (D)

Comparing mushrooms species, it may be observed that the extracts of *B.luridus* isolated with cyclohexane solvent possessed antibacterial activity against tested bacteria in the agar diffusion assay and showed largest clear active zones more than 15mm, however extract was not effective against *E. coli*. Also strong partial effect of *B. lepidus* (D), *B. impolitus* and *S. halophila* (M), against *S. aureus*; *G. ammophilus* (D, M) and *T.caligatum* (M) against *B. cereus*; *B. luridus* and *C. infractus* (M) against *P.aeruginosa*; *A. pseudoprattensis* against *E.coli* inhibited tested microorganisms in agar diffusion assay more than 15mm, but did not fully inhibit growth. Figure 3.6 represents the amount of mushroom extracts with clear active zones and partially inhibition zones against tested microorganisms. As mentioned above *B.luridus* (C), *P. schweinitzii* (D, M), *I. hispidus* (M) *H. ferrugineum* (M) showed strongest clear zones activity (>15mm) and inhibited *S. aureus*, *B. cereus*, *P.aeruginosa* bacteria respectively. Twelve extracts (*A. menieri* (D), *A. pseudoprattensis* (D), *B. impolitus* (M), *X. chrysenteron* (M), *T. caligatum* (D, M), *T.columbetta* (M), *A. ovoidea* (D), *C. infractus* (D, M) *L. vinosus* (D), *I. hispidus* (M)) showed activity against *P.aeruginosa*; six mushrooms fractions (*A. fissuratus* (D), *S. subvolvatus* (D), *B. impolitus* (C), *T. focale* (C), *A. ovoidea* (M), *C. infractus* (D)) possessed antioxidant activity against *S. aureus*; five extracts (*X. chrysenteron* (M), *T.columbetta* (D, M) *P. schweinitzii* (C), *I. hispidus* (M)) showed activity against *B.cereus* and two extracts (*B. lepidus* (M), *T. auratum* (M)) were active against *E. coli* with inhibition zone 10-15mm, other 55% extracts inhibit activity at range of 7-9mm. Otherwise *B.cereus* did not fully stop microorganism growth in agar diffusion assay, but inhibits partial 10-15mm zones and possessed activity for 17 mushroom species. Inhibition between 10-15 mm with incomplete growth demonstrated 15 mushroom extracts against *S. aureus*, and 17 extracts for *P. aeruginosa* as well as for *E. coli*.

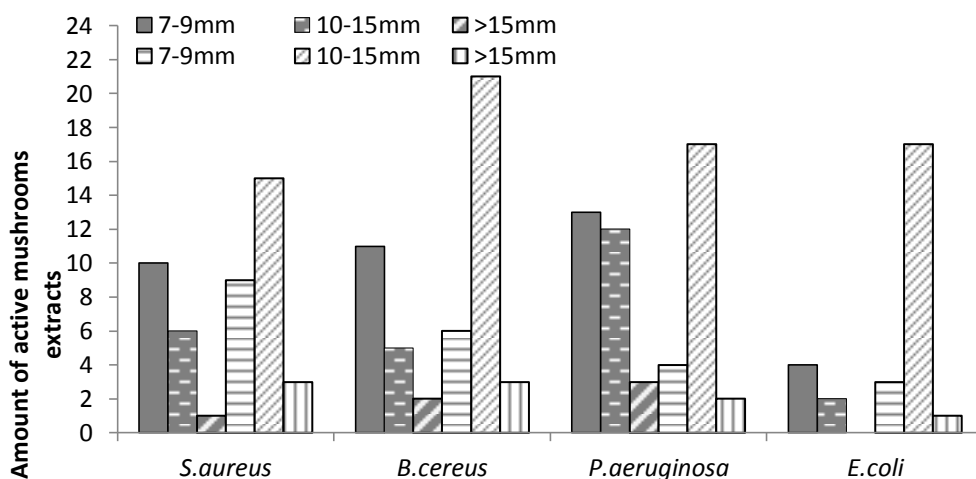


Fig. 3.6 Active amounts of mushroom extracts against tested microorganisms

MIC values which are evaluated by diluting the extracts and measuring the lowest inhibitory concentrations are important indicators of antimicrobial activity (Table 3.3). Five mushroom dichloromethane (*A. menier*, *A. pseudoprattensis*, *T. caligatum*, *C. infractus*, *L. vinosus*) and methanol extracts of *B. impolitus* were active against *P. aeruginosa*, four methanol (*G. ammophilus*, *B. lupinus*, *T. caligatum*, *A. ovoidea*) and dichloromethane extracts of *S. subvolvatus* and *C. B. luridus* were active against *S. aureus* and possessed MIC values 5 mg/mL. The lowest tested (5 mg/mL) MIC including partial activity in agar diffusion assay were determined 23 mushroom extracts against *B. cereus*, 15 extracts against *E. coli* and 25 mushrooms extracts against *P. aeruginosa*, as well as *S. aureus*. The majority of other extracts demonstrated MIC of 10 or 20 mg/mL.

Only few reports on antimicrobial activity of some species tested in our studies are available. Previously reported for *A. devoniensis* D and M extract were 10 mm and 20 mm inhibition zones against *S. aureus*; 8 mm and 10 mm against *P. aeruginosa*; 8 mm and 15 mm against *E. coli* respectively (Suay et al., 2000) In our studies D extract of *A. devoniensis* was not tested, although M extract demonstrated weaker activity and inhibition zones were 7-9 mm against *S. aureus* as well as *E. coli*, when *P. aeruginosa* and *B. cereus* were predominant. In reported studies MIC of *B. lupinus* methanol extract were 50 mg/mL against *S. aureus*, 25mg/mL against *P. aeruginosa*, and inactive against *E. coli* (Nikolovska-Nedelkoska et al., 2013), in our M extract *B. lupinus* MIC showed 5mg/mL against the same bacteria, and was inactive against *E. coli* and *P. aeruginosa*. The reason of dissimilarities could be the separate extraction methods, concentrations of microorganisms or dilutions of extracts also cultivar, harvesting time, climatic conditions and other factors. In recorded studies of *T. auratum* dichloromethane and ethanol extracts showed similar antimicrobial activity against *S. aureus* (<10 mm inhibition zones), although methanol used as solvent in this studies (Yamaç and Bilgili, 2006). In earlier studies noted similar antimicrobial activity of *C. odora* against *Bacillus brevis* with inhibition zones 7-9 mm of intact extract and 10-15mm of injured ethyl acetate extracts. (Stadler and Sterner, 1998) Other records was not demonstrated any antimicrobial activity of methanol extract of *C. odora* (Suay et al., 2000) and essential oil of *C. infractus* (Beattie et al., 2010).

3.3.2. Agar-overlay (TLC bioautography) technique on mushroom species

Methanol extracts were fractionated by TLC bioautography assay and active substances shown as clear zones on a violet background. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. Clear zones and effectiveness of the compounds can be deduced as death of cells. The results obtained from 30 methanolic mushroom species, were 17 extracts demonstrated potential activity are summarized in Table 3.4. Seven mushroom species were active against *B. cereus*; eleven extracts showed activity against *E. coli*; twelve mushroom species inhibit *P. aeruginosa*. Mushroom species which possessed antimicrobial activity of one active

fraction constitute 54%; whereas species indicated two active fractions compose 47% from all tested extracts. Species of *P. schweinitzii* and *I. hispidus* showed considerable active inhibition zones with two fractions against *E.coli* and *P. aeruginosa* (Fig. 3.7); thus other tested species have not possessed the evident and bright zones with one or two active fractions and lead to moderate antimicrobial activity. Differences in tested methanol fractions determined due to low concentration, origin and structure of active substance which influence the antimicrobial potential on TLC plate. Various secondary metabolites such as alkaloids, flavonoids, phenols of the mushrooms may be attributed to the presence of antimicrobial activity in agar overlay technique. However bioautography is a very convenient and simple way of screening natural sources, especially with significant potential of biological active constituents on pathogenic microorganisms. Although the separation of natural products sometimes can be the challenge, and if the separated amount is very less in maximum cases, usually it is necessary to develop a process which can detect a small amount of substances and measure the biological activity at the same time (Dewanjee et al.) Considering these problems bioautographic detection technique combined together with other antimicrobial assays are one of the solution.

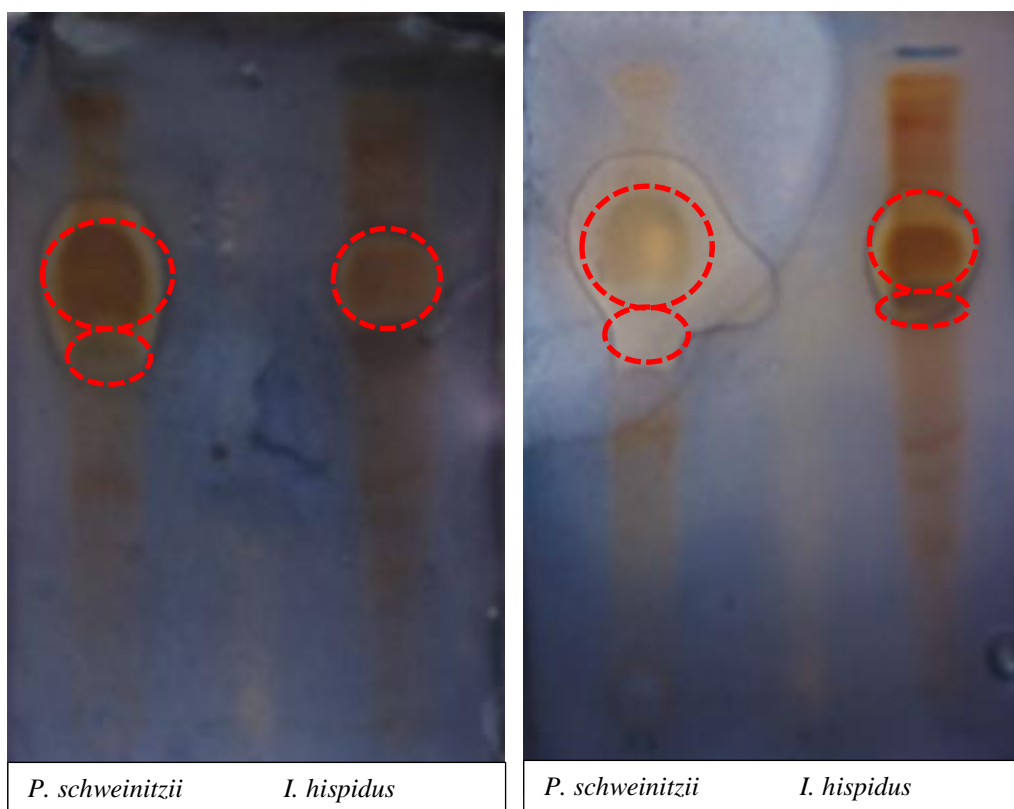


Fig. 3.7 *P.schweinitzii* and *I. hispidus* methanolic extracts inhibition zones against *E. coli* and *P. aeruginosa*

Table 3.4 TLC bioautography antibacterial evaluation of mushroom extracts

<i>Botanical name</i>	<i>Microorganisms</i>		
	<i>B. cereus</i>	<i>E. coli</i>	<i>P.aeruginosa</i>
<i>C. infractus</i>	+	+	++
<i>A. freirei</i>	-	+	++
<i>A. menieri</i>	-	-	+
<i>C. odora</i>	-	++	-
<i>G. ammophilus</i>	-	-	+
<i>P.schweinitzii</i>	+	++	++
<i>I. hispidus</i>	-	+	++
<i>H. ferrugineum</i>	-	+	-
<i>C. glaucescens</i>	+	+	+
<i>A. fissuratus</i>	+	-	++
<i>M. cinereifolia</i>	++	+	++
<i>A.coniferarum</i>	++	+	-
<i>T. auratum</i>	-	-	-
<i>S. halophila</i>	++	-	-
<i>A.ovoidea</i>	-	-	+
<i>A.devoniensis</i>	-	+	++
<i>C. suberetorum</i>	-	++	++
Control (methanol)	-	-	-

+ one active fraction; ++ two active fractions;

3.3.3. Biological properties of mushroom extracts¹

To identify new and potent antileishmanial agents, the mushrooms fractions were screened against the *L. infantum* amastigotes. Inhibitory concentration 50% (IC₅₀), cytotoxic concentration which reduced 50% of cell viability and selectivity index (SI) are reported in Table 3.5. Amphotericine B was used as control drug. The results obtained for 80 extracts isolated from 30 mushroom species only 21 species from which 43 fractions showed the activity. Ten fractions (*G. ammophilus* (C); *B.impolitus*(C); *B.luridus* (C, D, M); *S.halophila* (C, D); *C.suberetorum* (M); *C.infractus* (C, D)) displayed IC₅₀ values less than 10µM and could be considered as active as amphotericine B.

Generally, most active mushrooms were *G. ammophilus* (C) with IC₅₀ value 2.04µg/mL and *S.halophila* with IC₅₀ value 4.37 µg/mL (C), 4.46 µg/mL(D). Species *B.impolitus* (C), *B.luridus* (C, D, M); *C.infractus* (C, D) *C.suberetorum* (M) (IC₅₀ value 7.10, 8.60, 6.54, 8.58, 8.00, 9.81, 6.72µg/mL respectively) seemed to influence antileishmanial activity against amastigote. Species *A.freirei* (C,D); *L. litoralis* (C,D); *G.ammophilus* (D) *B.lepidus* (C,M); *B.impolitus* (D); *T.focale* (M,D); *T.caligatum* (C); *C.odora* (D); *C.suberetorum* (C) (IC₅₀ value 11.39, 10.89, 12.89, 15.17, 13.03, 12.03, 10.21, 14.00, 14.08, 14.55 and 12.74 µg/mL respectively) offered only moderate efficacy. Most of the other fractions remained weakly active in the range 15.17 µg/mL (*L. litoralis*, C) - 60.29 µg/mL (*A. pseudopratisensis*, D) or inactive on amastigotes form of *L. infantum*. The antileishmanial activity indicated 40% dichloromethane, 37% cyclohexane and 23% methanol extracts from all tested mushroom species.

¹Pharma Dev, Université de Toulouse, IRD, UPS

Table 3.5 *In vitro* antileishmanial activities against amastigotes of *L. infantum* and cytotoxicity evaluation

Botanical name	Fraction	Amastigote (<i>L.infantum</i>) IC ₅₀ µg/mL	Macrophage (cytotoxicity) CC ₅₀ µg/mL	SI (selectivity index)
<i>A. menieri</i>	C	21.83	>100	>4.58
<i>A. pseudopratisensis</i>	D	60.29	>100	>1.66
<i>A. freirei</i>	C	11.39	>100	>8.78
	D	10.89	>100	>9.18
	M	90.96	>100	>1.10
<i>A. devoniensis</i>	D	58.06	>100	>1.72
<i>A. coniferarum</i>	C	40.46	>100	>2.47
	D	31.76	>100	>3.15
<i>L. litoralis</i>	C	12.89	>100	>7.76
	D	15.17	74.25	4.89
<i>L. subolivaceus</i>	C	73.06	>100	>1.37
	D	43.20	>100	>2.31
<i>S. subvolvatus</i>	M	44.04	>100	>2.27
<i>G. ammophilus</i>	C	2.04	>100	>49.02
	D	10.18	>100	>9.82
<i>B. lepidus</i>	C	13.03	>100	>7.67
	M	12.84	>100	>7.79
<i>B. impolitus</i>	C	7.10	>100	>14.08
	D	10.21	>100	>9.79
	M	64.85	>100	>1.54
<i>B. luridus</i>	C	8.60	>100	13.21
	D	6.54	78.81	12.05
	M	8.58	>100	>11.68
<i>S. halophila</i>	C	4.37	>100	>22.88
	D	4.46	>100	>22.42
	M	46.66	>100	>2.14
<i>T. focale</i>	C	29.48	87.04	2.95
	M	14.00	>100	>7.14
<i>T. auratum</i>	D	14.08	23.12	1.64
<i>T. caligatum</i>	C	14.55	>100	>6.87
	D	20.22	>100	>4.95
	M	161.96	>100	>0.62
<i>C. odora</i>	C	17.64	>100	>5.67
	D	12.74	>100	>7.85
<i>C. suberretorum</i>	C	13.35	>100	>7.49
	D	16.59	>100	>6.03
	M	6.72	>100	>14.88
<i>C. infractus</i>	C	8.00	>100	>12.50
	D	9.81	>100	>10.19
<i>P. schweinitzii</i>	C	22.66	>100	>4.41
	D	22.72	>100	>4.40
<i>I. hispidus</i>	D	53.17	>100	>1.88
	M	61.00	>100	>1.64
Amphotericine B		0.28	30.94	110.5

C, cyclohexane; D, dichloromethane; M, methanol; Amphotericin B: reference drug for antileishmanial tests. IC₅₀ values (concentrations inhibiting parasite growth by 50%). CC₅₀ values (50% cytotoxicity concentration). ^aIC₅₀ and ^bCC₅₀ values are represented as average of at least duplicate measurements (SD 10%). ^cSI (selectivity index) = CC₅₀/IC₅₀.

To identify new and potent antileishmanial agents, the mushrooms fractions were screened against the *L. infantum* amastigotes. Inhibitory concentration 50% (IC₅₀), cytotoxic concentration which reduced 50% of cell viability (CC₅₀) and selectivity index (SI=CC₅₀/IC₅₀) are reported in Table 5. Amphotericin B was used as control drug. From the results obtained for 80 extracts isolated from 30 mushroom species, 21 species from which 43 fractions showed the activity (established as IC₅₀ < 25 µg/mL). Ten fractions (*G. ammophilus* (C); *B. impolitus*(C); *B. luridus* (C, D, M); *S. halophila* (C, D); *C. suberetorum* (M); *C. infractus* (C, D)) displayed IC₅₀ values less than 10 µg/mL and could be considered as very interesting extracts concerning their leishmanicidal potential.

Generally, most active mushrooms were *G. ammophilus* (C) with IC₅₀ value 2.04 µg/mL and *S. halophila* with IC₅₀ value 4.37 µg/mL (C), 4.46 µg/mL (D). Species *B. impolitus* (C), *B. luridus* (C, D, M); *C. infractus* (C, D) *C. suberetorum* (M) with IC₅₀ values less than 10 µg/mL seemed to influence antileishmanial activity against amastigote. Species *A. freirei* (C,D); *L. litoralis* (C,D); *G. ammophilus* (D) *B. lepidus* (C,M); *B. impolitus* (D); *T. focale* (M,D); *T. caligatum* (C); *C. odora* (D); *C. suberetorum* (C) with IC₅₀ values ranged between 11 and 14 µg/mL offered only moderate efficacy. Most of the other fractions remained weakly active (*L. litoralis*, C) (*A. pseudoprattensis*, D) or inactive to fight amastigote forms of *L. infantum*. The antileishmanial activity indicated 40% dichloromethane, 37% cyclohexane and 23% methanol extracts from all tested mushroom species.

The cytotoxicity influence on mouse macrophage cell line was evaluated for tested species in parallel because macrophages are the host cells of amastigotes in the definitive host organism. In order to identify fractions which were less toxic to mouse macrophage cell and at the same time selected as relevant clinical drug against *L. infantum* amastigotes. CC₅₀ values ranged from 23.13 (*T. auratum*, (D)) to more than 100 µg/mL for most of fractions (Table 5) which reduced 50% of cell viability. Only the four fractions *T. auratum* (D), *L. litoralis* (D), *B. luridus* (D), *T. focale* (C) had cytotoxicity less than 100 µg/mL (23.12, 74.25, 78.81 and 87.04 µg/mL respectively). The selectivity index (SI) was calculated as the ratio of cytotoxicity (CC₅₀ value on mouse cells) to activity (IC₅₀ value on amastigotes). This *in vitro* selectivity index showed that fraction *G. ammophilus* (C, SI=49) possessed the better selectivity profile than in research on species *A. blazei* water extract with lower selectivity index (SI=42) against *L. chagasi*, this mushroom use has been associated with folk medicine in the treatment of some diseases, like leukemia, cancer, and arterial hypertension (Valadares et al., 2012). Moderate efficacy selectivity index had *S. halophila* (C, D, SI=22, SI=22), in a contrast weaker activity possessed *C. suberetorum* (M, SI=14.88), *B. impolitus* (C, SI=14), *B. luridus* (C, SI=13), other species obtained very low selectivity index explaining that they were less suitable for parasite *L. infantum* or active compounds in fractions are in micro concentrations. Fractions which exhibited good to moderate antileishmanial activity have to be investigated to explain promising activities.

The results obtained for 90 extracts isolated from 30 mushroom species with described antibacterial and biological *in vitro* activity. 89% mushroom fractions

possessed antimicrobial activity and expressed in minimal inhibitory concentration with listing inhibition zones. The methanol extracts from *P. schweinitzii*, *I.hispidus* and *C. infractus* possessed most powerful antimicrobial activity in disc diffusion assay. The tested mushroom species showed greater antibacterial activity against Gram-positive than Gram-negative bacteria. Among the all tested mushroom extracts only 68% were biologically active as antileishmanial agents. Mushroom species of *G. ammophilus* (C), *S.halophila* (C, D), exhibited potent selectivity index.

Based on obtained data *P. schweinitzii* was chosen as research object for further detailed analysis.

3.5.ISOLATION AND IDENTIFICATION THE SECONDARY METABOLITES OF METHANOLIC EXTRACT FROM PHAEOLUS SCHWEINITZII (FR.)

In a comprehensive evaluation of wild mushrooms grown in Midi-Pyrénées region of France for their antioxidant, antimicrobial and biological properties, it was found that methanol extract of *Phaeolus schweinitzii* (Fr.) distinguished the strong capacity in all tested assays. Previously published data is particularly lacking main components, which are responsible for superior activity. The aim was identify the major compounds of methanolic extract of *Phaeolus schweinitzii* with assistance of chromatographic assays.

3.5.1. Detection of secondary metabolites by thin layer chromatography

In analytical practice thin layer chromatography (TLC) is still one of the commonly applied method for fast qualitative and quantitative analysis as well as screening of target substances from natural samples (Zarzycki et al., 2011).

TLC method gives quick access for localization, detection and category of the compounds in complicated fractions (Kaale, Risha and Layloff, 2011) A test was carried out on three silica gel 60 F₂₅₄ TLC plates (10.0 ×2.0 cm) in order to locate and select the category of compounds. First, 10µL of methanol extract was developed in the solvent system Chloroform/methanol/water (65:45:10). The TLC plates were then carefully dried; absorbing spots were detected at 254 and 366 nm and covered with three different reagents Fig. 3.8.

Two coloured zones were detected: their R_f values were found to be 0.5 and 0.85. On comparing results with TLC plates acquired in UV (254 and 366nm) and, a plate sprayed with Neu reagent (after heating 110 °C under UV 254 and visible light) for detection of flavonoids (Fig. 3.8, mode 2) and TLC plate observed in vanillin/sulfuric acid vapour (Fig. 3.8, mode 1) under visible light for detection of all organic metabolites such as steroids and terpenoids it was possible to establish the exact position of two active coloured substances. Dragendorff's reagent has not exposed the activity of alkaloids or substances with nitrogen atom (Fig. 3.8, mode 3) and orange-brown spots have not observed.

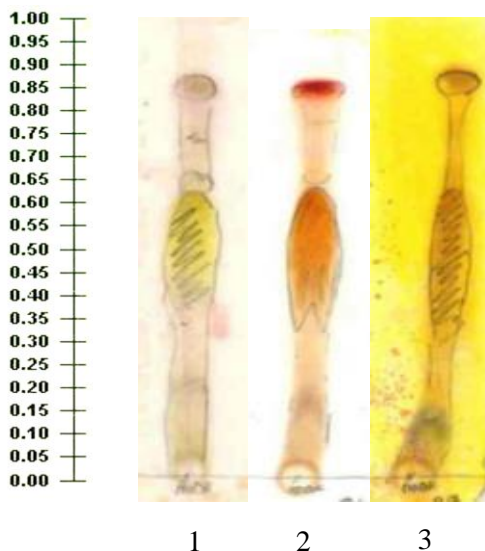


Fig. 3.8 *P. schweinitzii* methanol extract on TLC plates (visible light): detection mode (1) TLC exposed with vanillin/sulfuric acid; (2) TLC exposed with Neu reagent; (3) TLC exposed with Dragendorff reagent.

3.5.2. Detection of secondary metabolites by TLC-DPPH[•] bioautography

TLC-DPPH[•] assay belongs to a group of frequently performed methods aimed at detection of compounds with effective direct activity. Thin layer chromatography assay plates are developed and sprayed with DPPH[•] reagent in methanol for antioxidant screening. It enables visualisation of separated spots exhibiting antioxidant activities and localisation of potential substances (Głód et al., 2015). This method has several advantages: direct access to separated compounds, high throughput, flexibility, possibility to assess the activity of individual compounds found in complex samples, it also allows rapid dereplication of known active compounds and others (Cieśla et al., 2012). In the assay DPPH[•] scavenging activity was observed visually as white yellow spots on a purple background. *Figure 3.9* (modes 6 and 7) shows a profile of the antioxidant components in the methanol extract of *P. schweinitzii* under visible light. Two main spots $R_f=0.5$ and $R_f=0.85$ in the silica gel plates were observed to have DPPH[•] scavenging activities, which are identical in spite of their R_f values differing slightly between detection modes (*Fig. 3.9*). The same TLC plate was also inspected under visible light after 60 min (*Fig. 3.9* mode 7). Note that the same two antioxidant spots were observed, either other intangible substances exposed on TLC plate. However these differences exposed due to the diverse nature and capacity of the antioxidative compounds.

Ueno (Ueno et al., 1964) reported on the separation and isolation of hispidin from acetone extract of *P. schweinitzii*. A paper chromatographical investigation was used and hispidin possessed the $R_f=0.4$ value. Accordingly, in our research of

two antioxidatively active hispidine derivates in the methanol extract of *P.schweinitzii* was postulated.

3.5.3. Isolation of active substances from *P. schweinitzii*

Silica gel chromatography has been used for fractionating the methanol extract of *P. schweinitzii*. The extract impregnated with silica gel was subjected to silica gel chromatography using dichloromethane/methanol gradient elution (98:2, 95:5, 90:10,85:15, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100, v/v) give 52 fractions. Each fraction were tested with TLC silica gel chromatography and mainly combined to 7 fractions. Fraction 1 was chromatographed over Sephadex LH-20 eluted with methanol and further purified by preparative HPLC to afford compound 1 (3.9mg) and compound 2 (2.7mg). The fraction 3 was eluted with Sephadex LH-20 to obtain compound 3 (25.7mg) (Fig. 3.10).

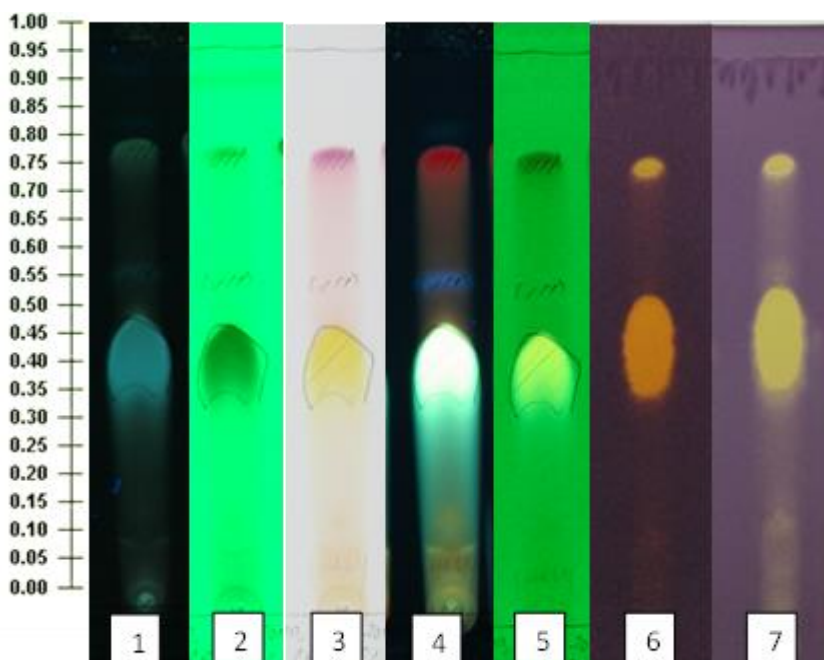


Fig. 3.9 *P. schweinitzii* methanol extract on TLC plate detection mode: (1) TLC under UV light 366nm; (2) TLC under UV light 254nm; (3) TLC exposed with Neu reagent under visible light; (4) TLC exposed with Neu reagent under UV light 366nm; (5) TLC exposed with Neu reagent under UV light 254nm; (6) TLC exposed with DPPH' reagent under visible light, after 1min; (7) TLC exposed with DPPH' reagent under visible light, after 30min

3.5.4. Purification and structure elucidation of active compounds

For further experiments fraction 1 of *P.schweinitzii* was used. In order to create preparative HPLC method for optimal isolation of active substances from methanol fraction, an analytical column XBridge 5- μ m (4.6 i.d. \times 150mm) was used. A wavelength of 377nm proved to be the optimal monitoring wavelength. The program

developed was scaled up successfully to preparative column XBridge 5- μm (19 i.d. \times 150mm).

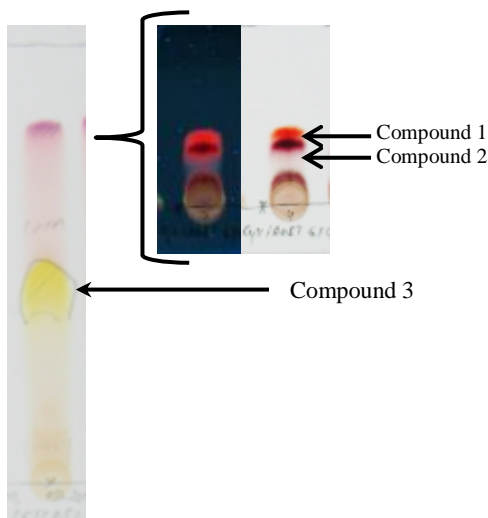
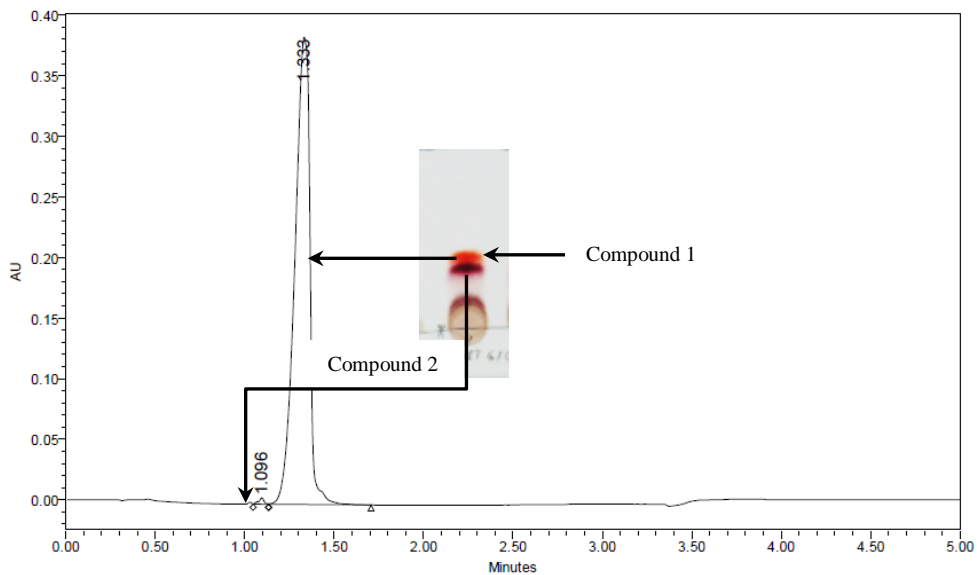


Fig. 3.10 *P. schweintzii* methanol extract on TLC plate under visible light; mode 1: TLC plate under UV 366 nm; mode 2: TLC plate under visible light.

Two main peaks were detected on the HPLC preparative chromatogram: 3.25 and 3.93 minute peak corresponded to the spot $R_f = 0.85$ and indicated two compounds. A 15 minutes run flow of 20mL/min was sufficient for separation of both substances. Both compounds were collected and concentrated under reduced pressure, after solvent residue removed with nitrogen flow. Isolates were re-subjected into UPLC-QTOF MS to check the purity and identify molecular mass in positive ion mode (Fig. 3.11 and 3.12). The separated compound 1 had UV adsorption maxima at 256 and 370nm, $R_t = 1.1\text{min}$ and compound 2 adsorption maxima at 243 and 377nm, $R_t = 1.3\text{min}$. The purity and quantity of isolated compounds was sufficient for further NMR and MS experiments.

MS (ESI in negative ion mode) and several NMR techniques (^1H , ^{13}C , H, H-COSY, HMBC and HSQC) were used for the structure elucidation of all isolated compounds.

For first NMR analysis, deuterated methanol was chosen to dilute all compounds. It is worth mentioning that improved structure decomposition was observed and described for all compounds in deuterated dimethylsulfoxide (DMSO).



	RT	Area	% Area	Height
1	1.096	11246	0.50	5349
2	1.333	2253351	99.50	385710

Fig. 3.11 QTOF MS chromatogram of *P. schweintzii* purified fraction, compound 2.

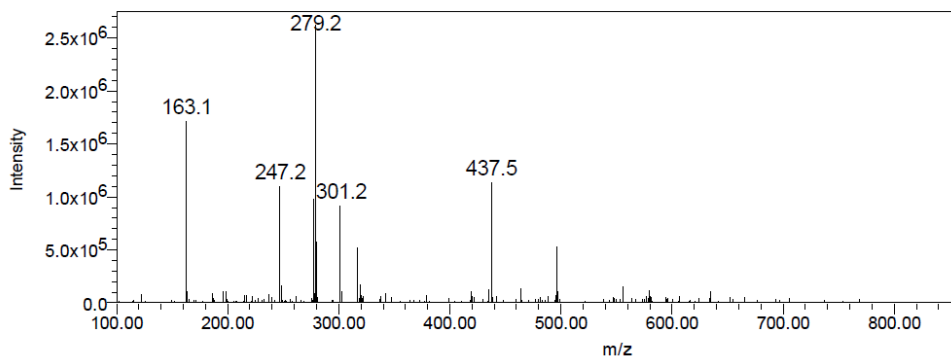


Fig. 3.12 MS⁺ spectra of *P. schweintzii* purified fraction, compound 2

3.5.5. Structure elucidation of hispidine (compound 3)

This compound was the major yellow powder from methanol fraction 3 of *P. schweintzii*. 27.5 mg of pure substance was gained from 2g methanol fraction and purified as described above. Its chemical composition was determined by high

resolution MS analysis. For ionization, electrospray (ESI) was used in negative mode. As can be seen from *Figure 3.13*, this high resolution negative ion mode ESI spectrum shows a characteristic ion series and compound fragmentation mechanism at 35.00V magnetic field. The ion $m/z = 245.05$ $[M-H]^-$, with molecular formula $C_{13}H_{10}O_5$ derived from ESI-MS/MS spectrum, which indicated fragment ions at $m/z = 201, 159$ and 135 corresponding to the proposed structures drawn in *Fig. 3.14*.

The 1H NMR spectrum indicated signals attributable to a trisubstituted phenyl moiety at δ 6.78 (1H, d, $J = 8.2$ Hz, H-13), 6.96 (1H, dd, $J = 8.2, 2.1$ Hz, H-14), 7.05 (1H, d, $J = 2.1$ Hz, H-10), a *trans* di-hydrogenated double bond at δ 6.70 (1H, d, $J = 16.0$ Hz, H-7) and 7.15 (1H, d, $J = 16.0$ Hz, H-8) and two constituents protons δ -lactone heterocyclic ring at δ 6.17 (1H, d, $J = 2.0$ Hz, H-5), 5.29 (1H, d, $J = 2.0$ Hz, H-3). Also three exchangeable hydroxyl phenolic protons at δ 9.21, 9.53, 11.0.

^{13}C NMR data analysis reveals the 13 carbon signals (from which oxygenated are C4 (δ 171.0), C11(δ 146.1), C12 (δ 147.9)) and a carboxyl carbon at δ 163.5 ppm. The proton signals following respectively with ^{13}C data (ppm): H7 (δ 6.70) with C6 (δ 160.2) and C5 (δ 101.0), H5 (δ 6.17) with C3 (δ 91.0), C4 (δ 171.0), C6 (δ 160.2) and C7 (δ 118.4), H3 (δ 5.29) with C2 (δ 163.5), C4 (δ 171.0) and C5 (δ 101.0) the spectra were identical to literature reports (Singh et al., 2003) and enabled the identification of 6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone, also known as hispidin (*Fig. 3.15*).

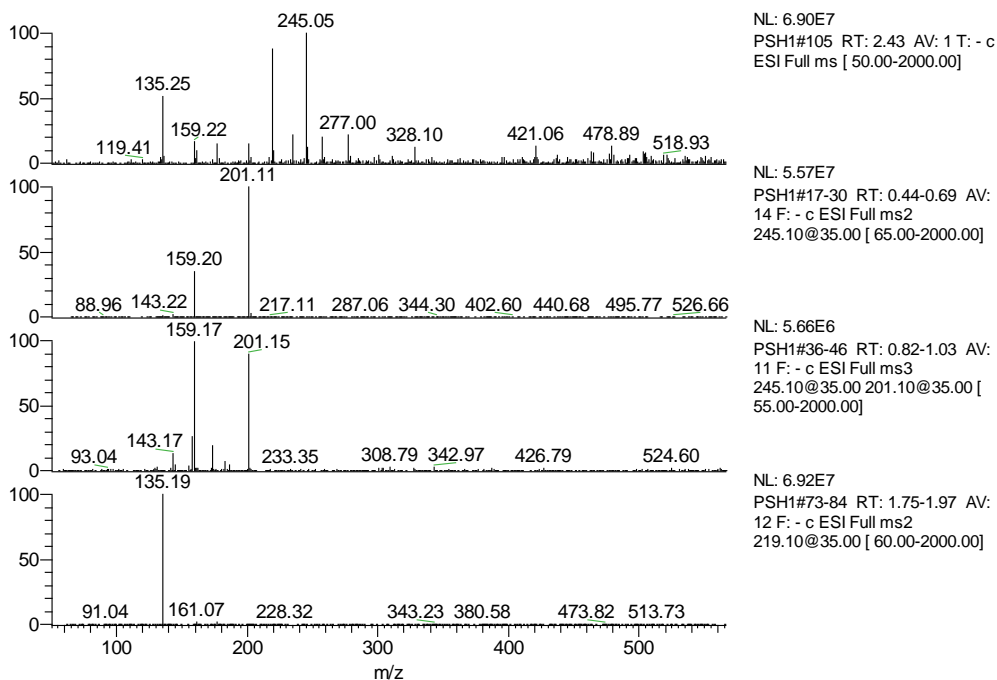


Fig. 3.13 Negative ESI spectra of compound 3 isolated from *P. schweinitzii* extract

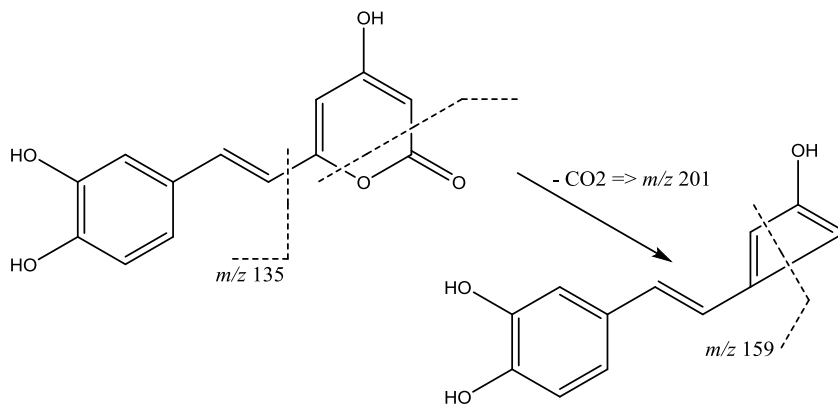


Fig. 3.14 Compound 3 (hispidin (6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone)) fragmentation mechanism isolated from *P. schweinitzii* extract

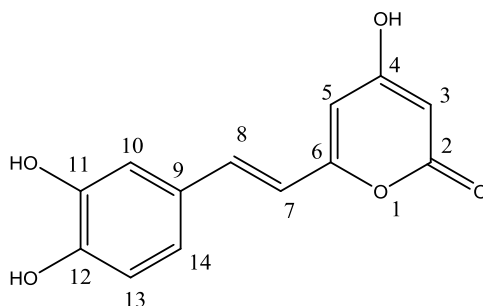


Fig. 3.15 Structure of hispidin (6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone, compound 3)

In literature hispidin was first detected in *Inonotus hispidus* (*Polyporus hispidus*) (Edwards, Lewis and Wilson, 1961) and the structure confirmed by Edward and Wilson (Edwards and Wilson, 1961). Afterwards it has been found in various related fungal species, such as *Inonotus xeranticus*, *Phellinus linteus* (Jung et al., 2008), *Gymnopilus spectabilis* (Lee et al., 2008b). Hispidin has been shown to possess antioxidant activity by inhibiting lipid peroxidation at rat liver homogenates (Khushbaktova et al., 1996, Lee and Yun, 2007, Park et al., 2004), to inhibit protein kinase C (PKC) and to possess cytotoxicity against cancer cells (Gonindard et al., 1997). Either, hispidin can protect against peroxynitrite-mediated cytotoxicity, DNA damage and hydroxyl radical formation (Chen et al., 2012).

3.5.6. Structure elucidation of hispolon (compound 1)

The pale yellow pigment can be observed on TLC with Neu reagent, resulting in an orange colour under visible light which indicates the presence of phenolics compounds and show a bright fluorescence under UV 366nm. Compared to hispidin, compound 1 shows very similar ^1H NMR signals of three aromatic and two *trans*-olefinic protons belonging to the styryl residue. The data of ^1H NMR spectra are listed as follows (ppm): 2.13 (3H, s, H-1), 5.80 (1H, s, H-4), 6.40 (1H, d, $J=15.7$ Hz,

H-5), 7.47 (1H, d, J=15.7 Hz, H-6), 7.05 (1H, d, J=2.1, H-8), 6.78 (1H, d, J=7.9Hz, H-11), 6.96 (1H, dd, J=2.1, 7.9 Hz, H-12) and exchangeable hydroxyl phenolic protons at δ 9.24, 9.56 the spectra were in correlation to literature reports (Wang et al., 2014b).

From the negative ion ESI MS spectrum it can be observed a characteristic ion series and compound 1 fragmentation mechanism at 35.00V magnetic field. The ion at $m/z = 219.1$ was interpreted $[M-H]^-$, with molecular formula $C_{12}H_{12}O_4$ and the ESI-MS/MS spectrum, of the $m/z = 219$ ion indicated the characteristic fragment ion at $m/z = 135$ base peak (Fig.3.16)

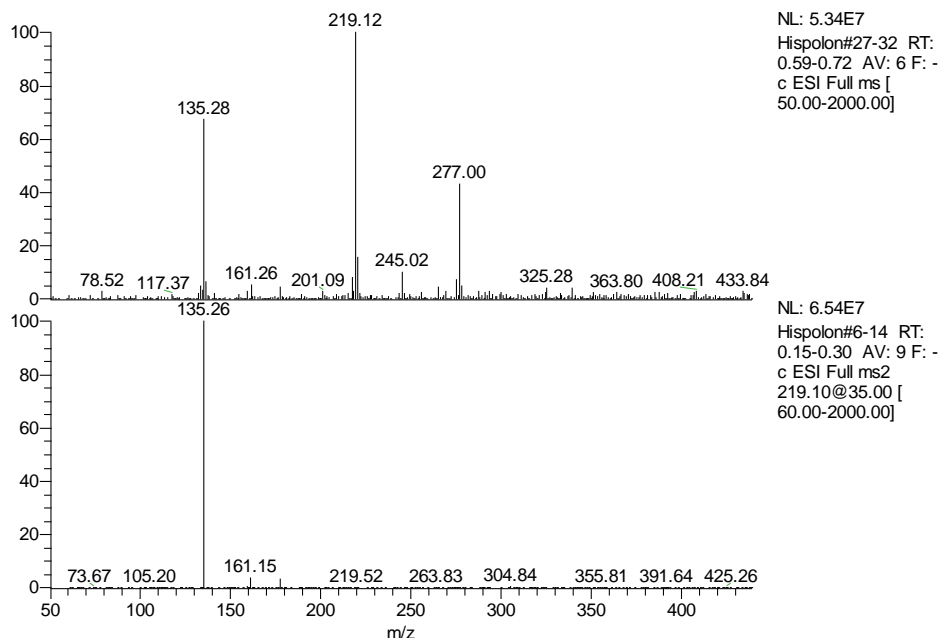


Fig. 3.16 Negative ion ESI spectrum and fragmentation pattern of compound 1 isolated from *P. schweinitzii* extract.

At this stage the data obtained for compound 1 were compared to the literature and were found to be congruent with the results published by (Ali et al., 1996) for a compound named hispolon (Fig. 3.17) (6-(3, 4-Dihydroxyphenyl)-4-hydroxyhexa-3,5-dien-2-one) firstly isolated from mushroom *Inonotus hispidus*. However, the

hispolon was first time isolated from *P. schweinitzii* in our research. Besides isolated from many *Phellinus* and *Inonotus* species (Mo et al., 2004, Wang et al., 2014b); hispolon (Fig. 3.17) were synthesized in several research groups (Venkateswarlu et al., 2002, Ravindran et al., 2010).

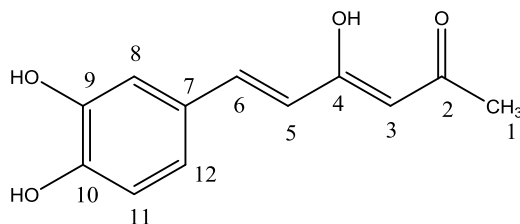
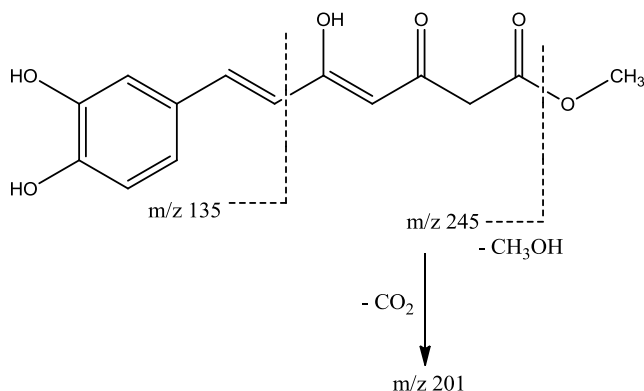


Fig. 3.17 Structure of hispolon (6-(3,4-Dihydroxyphenyl)-4-hydroxyhexa-3,5-dien-2-one, compound 1)

Hispolon has antioxidant, anti-inflammatory, analgesic and antiproliferative effects and inhibited cell growth or metastasis in various types of tumour cells (Mo et al., 2004, Chen et al., 2008, Chen, He and Li, 2006, Lu et al., 2010, Huang et al., 2011a). Further experiments reported that hispolon inhibits foot paw edema in mice (Chang et al., 2010). It protects against acute liver damage in rats by inhibiting lipid peroxidation, pro-inflammatory cytokine and oxidative stress (Huang et al., 2011b). Therefore, scientist suggest that hispolon has the potential to treat hyperpigmentation diseases and melanoma skin cancer in the future (Chen et al., 2014)

3.5.7. Structure elucidation of inonotic acid methyl ester (compound 2)

The yellow oily compound 2 can be observed on TLC plate with Neu reagent resulting in strong purple colour. The negative ion ESI MS spectrum showed molecular mass ion peak at $m/z=276.97$ $[M-H]^-$ in good agreement with molecular formula $C_{14}H_{14}O_6$ (Fig. 3.18). The ESI-MS/MS fragmentation of compound 2 indicated peak fragments ions at $m/z=245$ and $m/z=201$ similar to fragment ions previously described for hispidin.



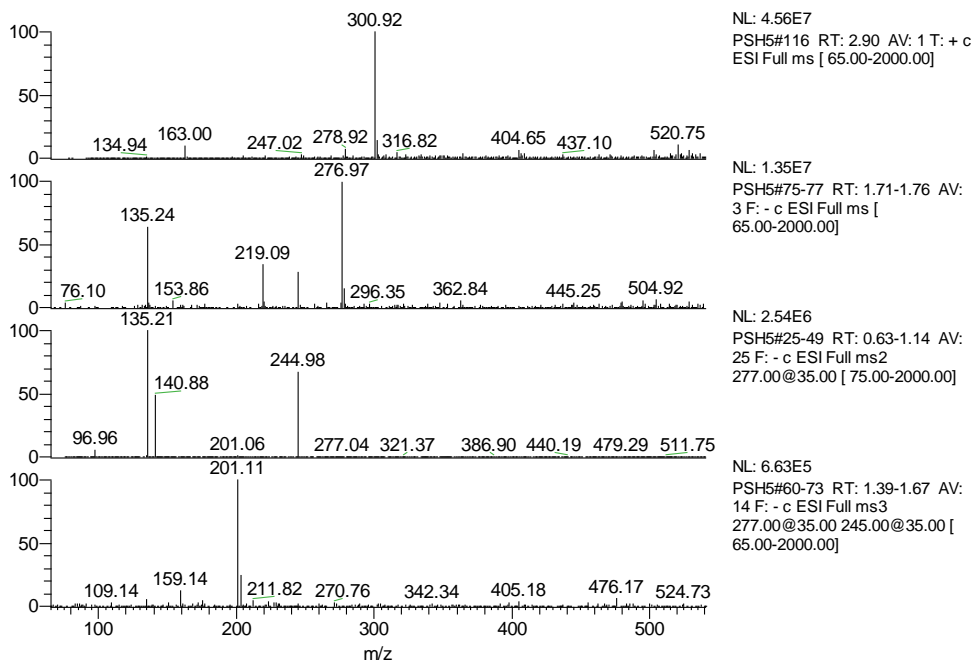


Fig. 3.18 ESI MS and ESI-MS/MS spectra and fragmentation pattern of compound 2 isolated from *P. schweinitzii* extract (from top to bottom: +ESI MS, -ESI MS, -ESI MS² of $m/z=277,-$ ESI MS³ of $m/z 277>243$).

The high-resolution mass spectrometry in positive ion mode established the chemical formula of compound 2 as $C_{14}H_{14}O_6$ with a molecular ion $[M+H]^+=279.0869$ and $C_{14}H_{14}O_6Na$ $[M+Na]^+=301.0688$. The ESI-MS/MS fragmentation of compound 2 indicated peak fragments ions at $m/z= 89.03, 107.05, 117.03, 135.04, 145.03, 163.04$ (Fig. 3.18).

The ¹H NMR also showed spectrum attributable to trisubstituted phenyl moiety at $\delta 6.77$ (1H, d, $J=8.0$ Hz, H-13), 7.00 (1H, dd, $J= 8.0, 1.8$ Hz, H-14), 7.07 (1H, d, $J= 1.8$ Hz, H-10), a *trans* di-hydrogenated double bond at $\delta 6.51$ (1H, d, $J= 15.8$ Hz, H-7) and 7.44 (1H, d, $J= 15.8$ Hz, H-8) as well as protons at $\delta 5.91$ (1H, s, H-5), 3.56 (2H, s, H-3) 3.65 (3H, s, H-1'). The ¹³C NMR spectrum indicated 14 carbons and following the analysis of the heteronuclear two-dimensional HSQC spectrum, it was possible to assign protons with their corresponding carbons and their multiplicity. The interpretation of the HMBC spectrum showed long range H-C correlations of H-10 ($\delta 7.07$) with C-8 ($\delta 141.08$), C-11 ($\delta 148.51$), C-12 ($\delta 145.67$), C-14 ($\delta 121.62$), the correlation of H-13 ($\delta 6.77$) with C-9 ($\delta 126.19$), C-11 ($\delta 148.51$), C-12 ($\delta 145.67$), C-14 ($\delta 121.62$) and the correlation of H-14 ($\delta 7.00$) with C-8 ($\delta 141.08$), C-10 ($\delta 114.73$), C-11 ($\delta 148.51$), C-13 ($\delta 115.82$) revealing an *ortho* substitution of the phenolic hydroxyl protons (Fig. 3.19). Also correlation of

H-8 (δ 7.44) with C-6 (δ 177.81), C-10 (δ 114.73), C-14 (δ 121.62) and other *trans* di-substituted proton H-7 (δ 118.62) correlated with C-6 (δ 177.81), C-9 (δ 126.19), C-13 (115.82). Proton H-5 (δ 5.91) correlation with C-6 (δ 177.81) and C-7 (δ 118.62). The spectra were correlated to literature reports (Wangun et al., 2006).

The other correlations illustrated in the *Figure 3.19* lead us to assign all atoms particularly methylene H-3 (δ 3.56) between the two keto groups C-2 (δ 168.01), C-4 (δ 192.03), C-5 (δ 100.56) and the position of the methoxy residue H-1' (δ 3.65) with C-2 (δ 168.01).

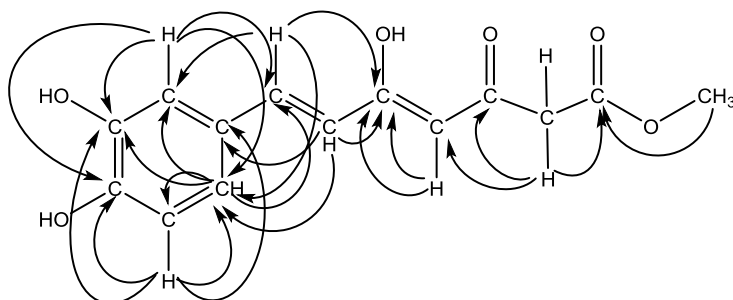


Fig. 3.19 HMBC associated protons to ^{13}C -atoms and their multiplicity of inonic acid methyl ester

Thus compound 2 was assigned as 7-(3,4-dihydroxyphenyl)-5-hydroxy-3-oxohepta-4,6-dienoic acid methyl ester (*Fig. 3.20*) and differs from hispolon by the presence of a carboxymethylester group fixed on the terminal Me of hispolon. Only one paper describes this compound, named as inonic acid methyl ester, isolation from *Inonotus* sp. with significant inhibitory activities against key enzymes involved in inflammatory processes (Wangun et al., 2006); however any information about this compound biological activity. Inonic acid methyl ester first time isolated from mushroom *P. schweinitzii* in our research.

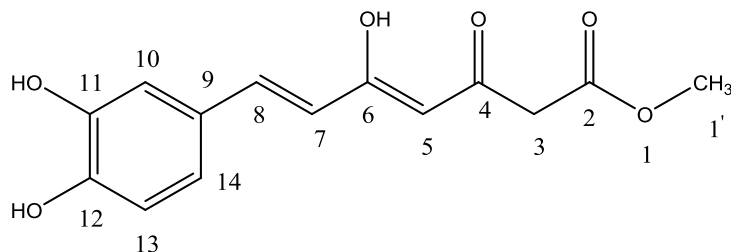


Fig. 3.20 Inonic acid methyl ester (7-(3,4-dihydroxyphenyl)-5-hydroxy-3-oxohepta-4,6-dienoic acid methyl ester, compound 2)

The structures of isolated metabolites share the same biosynthetic origin (*Fig. 3.21*). The compounds represent linear or cyclised polyketides derived from caffeoyl-CoA (Wangun et al., 2006) and may appear as biodegradation products.

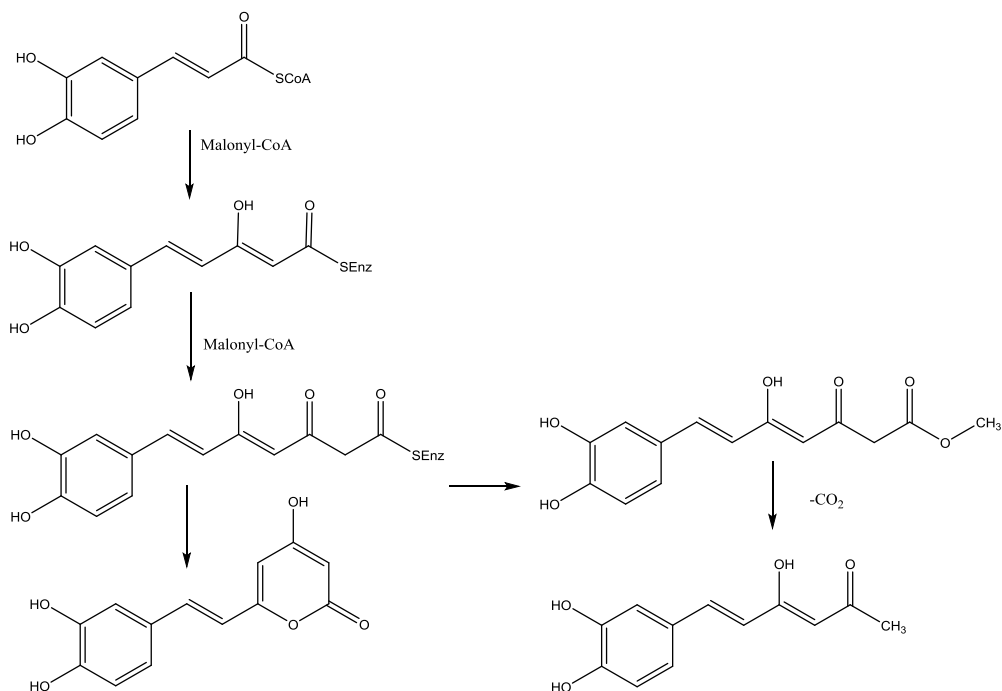


Fig. 3.21 *P. schweinitzii* metabolites and their biosynthesis

Three main compounds: hispidin, hispolon and inonic acid methyl ester isolated from methanolic extract of *P. schweinitzii*. Hispolon and inonic acid methyl ester in mushroom *P. schweinitzii* detected for the first time. Isolated compounds used for further analysis in order to evaluate antioxidant and cytotoxicity properties.

3.7. ASSESSMENT OF ANTIOXIDANT, BIOLOGICAL AND ANTIPARASITIC ACTIVITY OF ISOLATED COMPOUNDS FROM *P. SCHWEINITZII*

The search for novel bioactive molecules has been an ongoing task as long as we believe that pharmacologically active molecules are the answers for various human and animal diseases. In the last decade, the emergence of the modern pharmaceutical industry and the development of the wide range of effective medical and veterinary treatments trended to focus on natural resources. For example, the molecules derived from natural sources with possible protector agents reducing oxidative damage, which also may possess microbial, pharmacological or

antiparasitic activity. These active compounds with “multi-functional” properties may have potential implication in industry.

The aim was to determine isolated major compounds antioxidant and biological activities in several assays. Also to examine the influence of *P. schweinitzii* and *I. hispidus* extracts collected at different mushroom harvesting periods and their isolated compounds inhibitory activity properties against gastrointestinal nematodes *in vitro*.

3.7.1. Compound hispidin antioxidant activity

The evaluation of the antioxidant activity of the isolated compound hispidin was performed towards on different types of methods - single electron/hydrogen atom transfer assays (SET or HAT) used to obtain the desired results.

Determination of antioxidant capacity with ORAC-FL assay

The peroxy radical scavenging activity of the isolated hispidin was evaluated by the oxygen radical absorbance capacity (ORAC) method. This assay uses a fluorescence-based technology (ORAC-FL) and allows obtaining a relative antioxidant index by using as reference Trolox, a hydrosoluble vitamin E derivative.

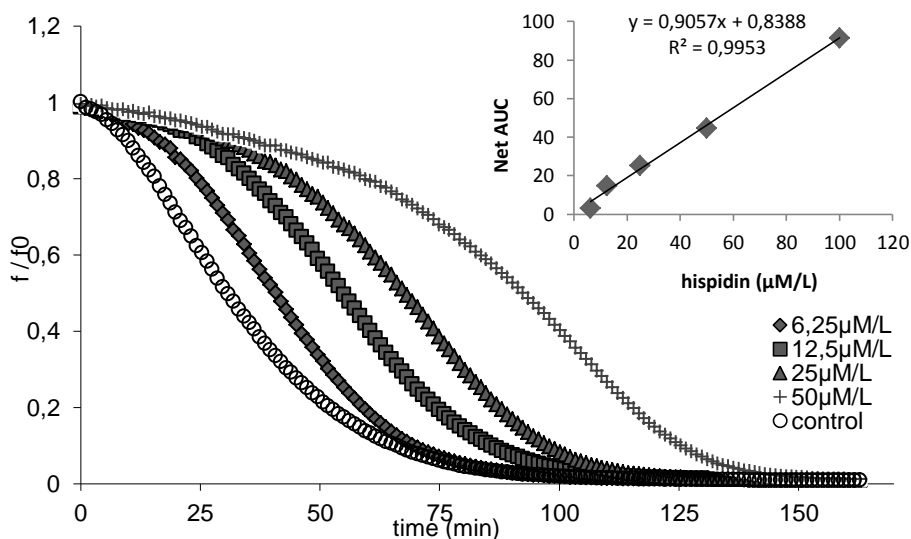


Fig. 3.22 ORAC-FL decay curves induced by AAPH in the presence of hispidin at concentration 6.25-50μM/L. Insert: NetAUC vs. hispidin concentration graph

The exposition of the fluorophore, in this case fluorescein (FL) to the peroxy radical conducts to an oxidation process reflected as a decay of fluorescence emission through time. In ORAC assays, the loss of fluorescence of FL generally corresponds to an induction time and is reliant on antioxidant capacity of a compound (Bisby, Brooke and Navaratnam, 2008). Indeed, it refers to the time in which the FL is

protected against the oxidative damage of peroxy radicals and this behaviour is associated to a competitive reaction between the radical and the antioxidant. ORAC data take into account the induction time, initial rate and the range of total antioxidant inhibition in one value.

Figure 3.22 and 3.23 displays the results in terms of the respective areas under the curve: The kinetic profiles obtained for Trolox (Fig. 3.23) were smaller than those obtained for hispidin (Fig. 3.22). The ORAC values were obtained by plotting Net AUC vs. concentrations of hispidin (Fig. 3.22 insert). The presence of induction time in ORAC-FL profile by addition of hispidin concentrations (6.25-50µM/L) was shown in Fig. 3.22. This is related to the time in which the probe molecule, in this case, Fluorescein, is protected against oxidation produced by peroxy radicals, in the presence of increasing amounts of antioxidant molecule - hispidin. Fig. 3.24 shows better hispidin kinetic profile than Trolox and has stronger oxidation protection stability.

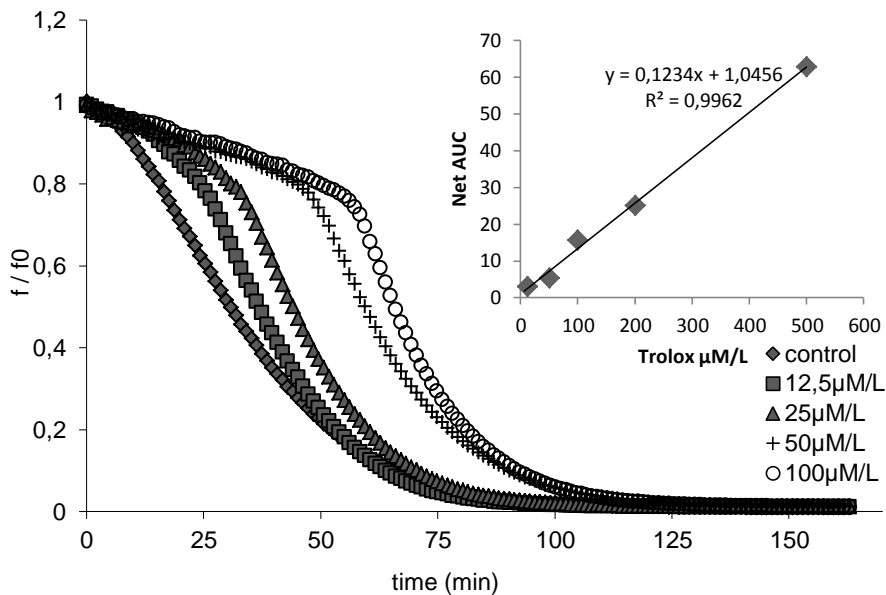


Fig. 3.23 ORAC-FL decay curves induced by AAPH in the presence of Trolox at concentration 6.25-50µM/L Insert: NetAUC vs. Trolox concentration graph

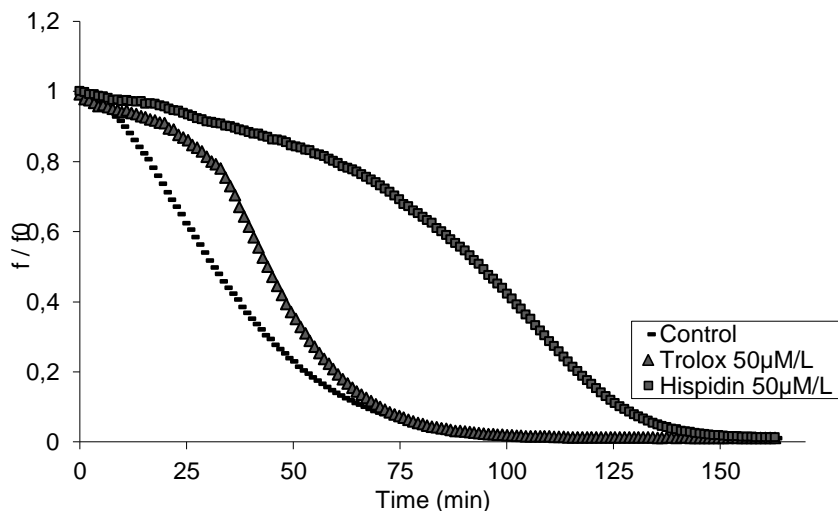


Fig. 3.24 ORAC-FL decay curves induced by AAPH in the presence of Trolox and hispidin

Determination antioxidant capacity with DPPH[•] assay

DPPH[•] assay is considered an easy and accurate method, appropriate for measuring the antioxidant capacity in several chemical analyses. This is due to the electronic properties shared by DPPH[•] and peroxy radicals (the unpaired electron is delocalized through the pair of nitrogen or oxygen atoms, respectively), in such way that the reaction rate between DPPH[•] and several antioxidants provides a good approximation for scavenging activities (Brand-Williams et al., 1995).

Figure 3.25 shows the percentage of DPPH[•] radical-scavenging activity of the samples, in comparison with the Trolox control. Note that the hispidin and Trolox showed a similar and even better antioxidant activity and increased with higher concentration. Whereas, Trolox reduction absorbance activity varied between 2.82-83.04% and hispidin between 3.32-87.53%. The half maximal inhibitory hispidin concentration needed to reduce DPPH[•] radical by 50% was measured - 547 ± 7.77 $\mu\text{M/L}$.

In reported studies DPPH[•] radical-scavenging activity of hispidin varied from 27-75% and showed better activity in smaller concentration from 25-200 μM respectively, than in our studies (Han et al., 2013). These dissimilarities could be dependent on DPPH[•] radical and hispidin ratio used in reported studies, which was 1:1 in our studies and 1:40 determined by Han (Han et al., 2013). Hispidin isolated from the cultured broth of the medicinal fungi *Inonotus xeranticus* and *Phellinus linteus* exhibited strong antioxidant activity and TEAC were 1.31 ± 0.81 expressed as IC_{50} of μM compound/ IC_{50} of μM Trolox (Jung et al., 2008), meanwhile the same research group isolated hispidin from mushroom *Gymnophilus spectabilis* and described as potent scavenging activity compound were TEAC 0.31 ± 0.81 expressed as IC_{50} of μM compound/ IC_{50} of μM Trolox (Lee et al., 2008b).

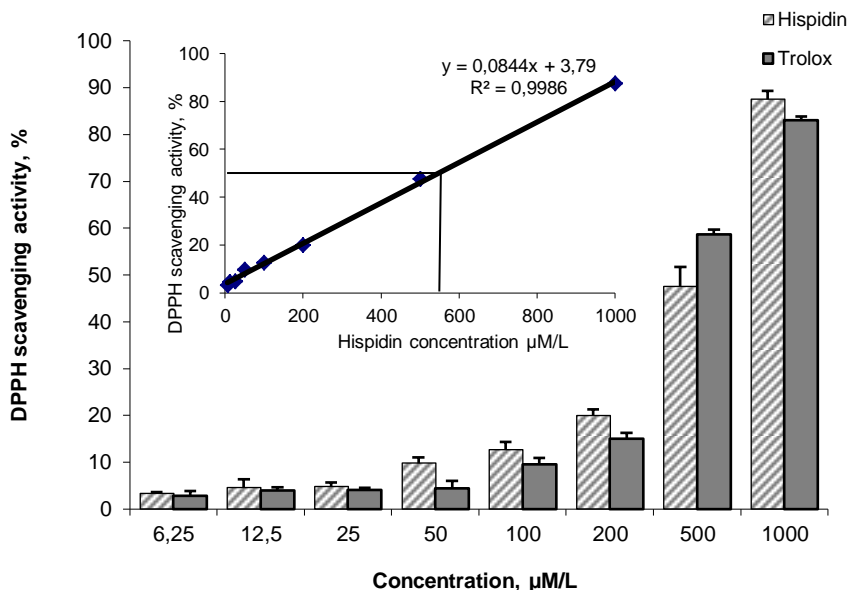


Fig. 3.25 Reduction absorbance (%) of DPPH[•] radical scavenging activity of hispidin and Trolox. Insert: Reduction absorbance vs. hispidin concentration graph

In our research recalculated DPPH[•] scavenging activity of hispidin and express as IC₅₀ of µM compound/IC₅₀ of µM Trolox TEAC=0.99±0.06, which is 75% less and three time more than define in different literature. Other source characterize hispidin glucopyranoside from Sword brake fern (*Pteris ensiformis* Burm.), the most common ingredients of traditional herbal drinks in Taiwan. The hispidin 4-O-b-D glucopyranoside has the same chemical structure that the glucose is linked at C-4 of hispidin. Hispidin glucosyde displayed stronger or equal DPPH[•] scavenging activities (28.21±1.51µM for reduction 50% DPPH[•] radical) as compared with the common antioxidant supplement, a-tocopherol were IC₅₀ = 28.08±2.69 51µM (Chen et al., 2007).

This potential activity indicates that the structure prerequisite to reinforce DPPH[•] scavenging is catechol moiety, of hispidin. In addition to OH moieties in the structural arrangements and the resonance of electrons between rings may also be important factor for their DPPH[•] scavenging activities.

Determination antioxidant capacity with ABTS^{•+} assay

The method used was based on the capacity of a hispidin to inhibit the ABTS^{•+} radical and compared with a reference antioxidant standard Trolox. During the ABTS^{•+} assay, the decrease in absorbance was followed to monitor the consumption of the coloured ABTS^{•+} radical.

The *Figure 3.26* shows ABTS^{•+} antioxidant capacity of hispidin and standard Trolox. Hispidin antioxidant capacity (varied 1.57-73.83%) was twice better than

standard Trolox (varied 1.20- 31.88%) in bigger concentrations, thus reduction absorbance with smaller concentrations was similar. The maximal inhibitory concentration needed to inhibit 50% of ABTS radical is $688 \pm 4.68 \mu\text{M/L}$ of hispidin.

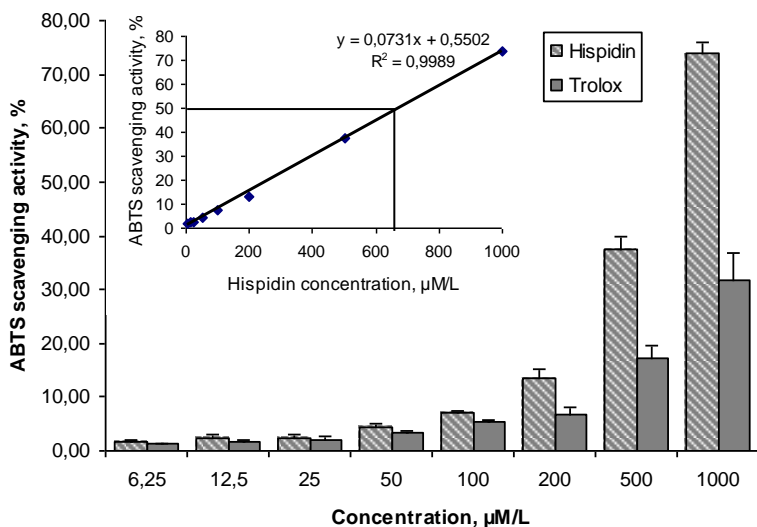


Fig. 3.26 Reduction absorbance (%) of $\text{ABTS}^{+\cdot}$ antioxidant capacity of hispidin and Trolox. Insert: Reduction absorbance vs. hispidin concentration graph

The hispidin isolated from *I. xeranticus*, *P. linteus* and *G. spectabilis* were able to scavenge the $\text{ABTS}^{+\cdot}$ radical cation and the activity were 2.72 ± 0.71 expressed as IC_{50} of μM compound/ IC_{50} of μM Trolox (Jung et al., 2008, Lee et al., 2008b); thus in our research recalculated $\text{ABTS}^{+\cdot}$ activity were lower more than three times ($\text{TEAC} = 0.76 \pm 0.08$ expressed as IC_{50} of μM compound/ IC_{50} of μM Trolox). Contrary $\text{ABTS}^{+\cdot}$ scavenging activity concentration of Trolox having the antioxidant equivalent to 1.0 mM of the hispidin ($1.66 \pm 0.14 \text{ mM}$) in our research was twice higher than hispidin glucoside ($0.85 \pm 0.04 \text{ mM}$) evaluated from plant *P. ensiformis* (Chen et al., 2007); It is worthy of noting that hispidin have part of caffeoyl moiety were are hydroxyl groups *para* and *ortho* substituted on an aromatic ring. These similarities may have potent antioxidant activity of hispidin. The similarities with caffeic acid (3,4-dihydroxycinnamic acid) which are identified as one of the active antioxidant explain the potent activity of hispidin (Gülçin, 2006).

Determination antioxidant capacity on FRAP assay

The ferric reducing antioxidant power (FRAP) assay measures the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) in the presence of antioxidants, which are reductants with half-reaction reduction potentials above $\text{Fe}^{3+}/\text{Fe}^{2+}$ (Gliszczyńska-Świąło, 2006).

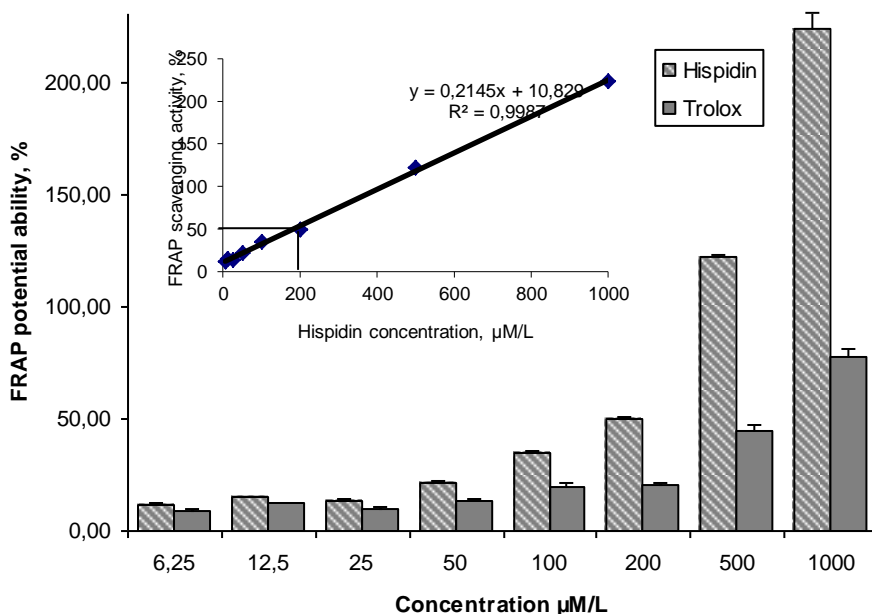


Fig. 3.27 Reduction absorbance (%) of FRAP reducing antioxidant power of hispidin and Trolox. Insert: Reduction absorbance vs. hispidin concentration graph

In the FRAP assay hispidin revealed the potential ability to reduce Fe^{3+} to Fe^{2+} , whereas activity varied between 11.70- 223.83%, and trolox 8.77- 77.93% (Fig. 3.27). More than half better activity comparing with Trolox. The maximal inhibitory concentration needed to reduce 50% of Fe^3 radicals is $194.37 \pm 3.28 \mu\text{M/L}$ of hispidin.

By using DPPH^{\bullet} radical, $\text{ABTS}^{+\bullet}$ radical cation, FRAP, ORAC oxygen adsorption capacity were assessed the free radical scavenging efficacy of hispidin. The results were expressed in maximal inhibitory concentration needed to inhibit 50% free radicals. Although the antioxidant activity of hispidin is known, its antioxidant single electron and hydrogen atom transfer assays yet to be determined. DPPH^{\bullet} and $\text{ABTS}^{+\bullet}$ scavenging activity data parallel described with literature source, FRAP and ORAC data estimated for the first time.

The antioxidant efficacy of hispidin may originate from the catechol moiety. It has been recommend that a good antioxidant must have low O-H bond dissociation enthalpy to simplify the H-abstraction from the radical and the hydrogen-transfer process must be stable, which is generated by radical (Zhang, Sun and Wang, 2003). Lower bond dissociation enthalpy value contributes to the O-H group in the ortho-diphenol moiety also stabilizing the radical form and has possible changes in the antioxidant capacity (Wright et al., 2001). Similarly antioxidant activity may be

affected by the intra-molecular hydrogen bonds that can exist in the catechol moiety and the inter-molecular hydrogen bonds between functional groups that involve polar solvents. In all tested assays hispidin, with catechol moiety exhibited similar or more potent activity than Trolox. Results show that hispidin was an effective antioxidant in different *in vitro* antioxidant assays.

3.7.2. Isolated compounds and *P. schweinitzii* and *I. hispidus* extracts biological activity

3.7.2.1. Determination of antileishmanial and cytotoxicity activity of isolated compounds ²

Leishmaniasis is one of the world most fatal diseases which occur in 98 mainly tropical countries. 350 Million peoples are considered at risk of contracting leishmaniasis, and over 2 million new cases occur each year. (Marín et al., 2013). Leishmaniasis caused by kineto-plastid protozoan infection from the *Leishmania* genus parasites that affect both human, other mammal species and transmitted through sandflies (Elmahallawy et al., 2014). Only a limited number of effective antileishmanial agents are available for chemotherapy (sodium stibogluconate, pentostam, meglumine antimoniate, glucantime, amphotericin B, pentamidine, paromomycin) and many of them are expensive with several side effects or have a markedly reduced effectiveness due to the development of drug resistance. Thus, the development of novel less toxic antileishmanial drugs is an important goal. In this concept, several compounds including synthetic ones, or natural products extracted from plants or marine sources, have shown different degrees of efficacy in the treatment of experimental leishmaniasis (Marín et al., 2013) (Machado et al., 2012).

In consequence of matter, isolated compounds from *P. schweinitzii* was evaluated the antiproliferative *in vitro* activity against of *L. infantum* promastigote forms. In parallel cytotoxicity influence on mouse line was evaluated for hispidin, hispolon and inonotic acid methyl ester. Amphotericine B was chosen as antileishmanial reference-drug, Doxorubicin used as positive control of cytotoxicity. All molecules were first screened *in vitro* on the promastigote stage of *L. infantum* by determining their inhibitory concentrations 50% (IC₅₀) and comparing them to the one of amphotericin B. In order to assess their selectivity of action, the tested molecules were also evaluated *in vitro* as regards of their cytotoxicity, measured by the cytotoxic concentrations 50% (CC₅₀) on the murine J774A.1 macrophages cell line, giving access to the corresponding selectivity indexes (SI = CC₅₀ / IC₅₀). Results are presented in *Table 3.6*.

Table 3.6 Compounds antileishmanial and cytotoxicity activity

	<i>L. infantum</i> promastigotes IC ₅₀ , (μM) ^a	J774A.1 CC ₅₀ , (μM) ^a	IS (CC ₅₀ /IC ₅₀)
Hispidine	76.92 ± 13,16	>100	>1,3

² Pharma Dev, Université de Toulouse, IRD, UPS

	<i>L. infantum</i> promastigotes IC ₅₀ , (μM) ^a	J774A.1 CC ₅₀ , (μM) ^a	IS (CC ₅₀ /IC ₅₀)
Hispolone	55.03 ± 3,18	18.84 ± 0,28	0,34
Inonotic acid methyl ester	53.88 ± 4,17	28.48 ± 1,42	0,53
Amphotericin B^b	0,03 ± 0,01	2.47 ± 0. 24	82,33
Miltefosin^b	8.83 ± 2.77	155.63 ± 15.25	17.63
Pentamidine^b	0.51 ± 0.28	0.53 ± 0.57	1.04
Doxorubicin^c	-	0.06 ± 0,04	-

a: Mean of three independent experiments; b: Miltefosine, Pentamidine (Paloque, Hemmert, Valentin & Gornitzka, 2015) and Amphotericin B, were used as antileishmanial compounds of reference; c: Doxorubicin was used as positive control of cytotoxicity.

The tested compounds exhibited moderate antileishmanial activity IC₅₀ in following order: Inonotic acid methyl ester < hispolone < hispidin. Thus compared to the cytotoxicity of Amphotericin B (2.47 ± 0.24 μM) or Pentamidine (0.53 ± 0.57 μM) compounds were from 7-12 times less toxic than referenced drug and globally slightly toxic. By comparing the selectivity index of referenced drug Pentamidine (1.04) the molecules were 33 and 51% less selective for hispolone and inonotic acid methyl ester respectively, contrary than more selective hispidin (25%). However tested compound had very low selectivity index compared to Amphotericin B or Miltefosin.

Represented data show that compounds may demonstrate an activity against on promastigotes - *L. infantum* specific target or that it has immunomodulatory effects modifying the antiparasitic response of infected macrophages. There have been no reports on antileishmanial activity of isolated compounds; however hispidin and hispolon isolated from ethanolic extract of *I. hispidus* showed considerable antiviral activity against influenza viruses type A and B (Awadh Ali et al., 2003). Hispidin isolated from *P. linteus* inhibit neuraminidases which catalyze hydrolysis of terminal neuraminic-acid residues from newly formed virions and from host cell receptors and are involved in release of progeny virus from infected cells (Yeom et al., 2012). The neuroprotective efficacy of hispidin (10–20 lg/mL) from the same mushroom was found to significantly inhibit peroxynitrite-induced DNA damage and cytotoxicity (Chen et al., 2012). Furthermore, several reports have demonstrated that hispolon exhibits anticancer effects through the inhibition of cell growth or metastasis in various types of tumour such as gastric, acute myeloid leukemia and human hepatoma cancer cells (Chen et al., 2008, Hsiao et al., 2013, Huang et al., 2010). The latest efficacy represent of hispolon could be used for treating the estrogen deficiency-related disease with the benefit of non-toxic to normal cells, good antitumor effects and estrogenic activity (Wang et al., 2014b). Overall hispidin, hispolon and inonotic acid methyl ester may represent a new lead scaffold for various drug developments, and thus its efficacy and side effect deserves further evaluation.

3.7.2.2. Detailed biological activity of mushroom metabolite hispidin³

The present study was to evaluate detail genotoxic activity of the hispidin isolated from *P. schweintzii*. As biomarkers of genotoxic effects, frequencies of gene mutations in *S. typhimurium*, as well as MN, CA, and SCE's and the extent of primary DNA damage detected by comet assay were used.

3.7.2.2.1. Induction of micronuclei in human lymphocytes *in vitro*

Lymphocytes from two donors were used for this part of the study and the results are shown in *Table 3.7*. Hispidin was not genotoxic in the micronucleus test at the dose range of 10–150 µg/mL. However, hispidin induced a slight though statistically significant decrease of nuclear division index (NDI) values in lymphocytes of both donors (*Table 3.7*). The highest tested dose (200 µg/mL) was cytotoxic.

Table 3.7 Micronucleus frequency (MN/1000 binucleated cells) and nuclear division index (NDI) values in peripheral blood lymphocytes after treatment with fungal metabolite hispidin *in vitro*

Treatment	Concentration (µg/mL)	Donor F1		Donor M1	
		MN, mean±S.E.M.	NDI, mean±S.E.M.	MN, mean±S.E.M.	MN, mean±S.E.M
Blank		3±1.73	1.50±0.04	3±1.73	1.57±0.04
Ethanol	7,5 µl/mL	3±1.73	1.64±0.04	3±1.73	1.72±0.04
Hispidin	10	–	–	4±1.99	1.62±0.04
	20	–	–	5±2.23	1.59±0.04*
	40	–	–	6±2.44	1.55±0.03*
	50	4±1.99	1.65±0.03*	5±2.23	1.71±0.04
	100	6±2.44	1.30±0.02*	6±2.44	1.55±0.03*
	150	8±3.18	0.92±0.01*	5±2.23	1.26±0.02*
	200	Cytotox.	Cytotox.	Cytotox.	Cytotox.

* P<0,05 when compared with vehicle (ethanol) controls.

3.7.2.2.2. Induction of chromosome aberrations and SCEs in human lymphocytes *in vitro*

We used lymphocytes from donor F2 for the studies of hispidin genotoxicity using two additional cytogenetic endpoints – chromosome aberrations (CAs) and sister chromatid exchanges (SCEs). Hispidin did not induce significant increase in chromosome aberrations (*Table 3.8*). However, it revealed genotoxic effect in SCE test. Hispidin induced statistically significant dose-dependent increase in SCE frequency ($y=9,5334+0,05613 \cdot x$; $r=0.98593$, $P<0,05$) and decrease in replication index (RI) values ($y=1.6887-0.0023 \cdot x$; $r=-0.8329$; $P<0,05$).

³ ¹ Experiments were performed at Department of Botany and Genetics, Vilnius University.

Table 3.8 Effects of hispidin extract on the frequency of chromosome aberrations (CA), sister chromatid exchanges (SCE) and replication index (RI) values in human lymphocyte cultures *in vitro*

Treatment	Concentration (µg/mL)	Chromosome aberrations ^a				Sister chromatid exchanges	
		CA per 100 cells (±S.E.M.)	ctb	cte	csb	SCE/cell±S.E.M.	RI±S.E.M
Blank	0	3.33±1.89	2	0	1	9.52±0.55	2.67± 0.04
Ethanol	7,5 µl/mL	3.23±3.12	1	0	0	9.94±0.47	2.67± 0.04
Hispidin	10	3.95±1.69	2	1	0	9.82±0,56	2.30± 0.05 ^b
	20	3.92±2.72	0	0	2	10.80±0.57	2.06± 0.06 ^b
	40	6.67±4.56	1	0	1	11.36±0.59	2.07± 0.06 ^b
	50	1.85±1.83	1	0	0	12.80±0.68 ^b	2.25±0.05 ^b
	100	5.89±4.04	1	1	0	14.32±0.53 ^b	2.19±0.05 ^b
	150	4.76±3.29	1	0	1	18.46±0.86 ^b	2.04± 0.05 ^b

^a ctb, chromatid breaks; cte, chromatid exchanges; csb, chromosome breaks; cse, chromosome exchanges. ^b P<0.05 as compared to adequate solvent control.

3.7.2.2.3. Induction of primary DNA damage detected by comet assay

The Comet assay was performed on isolated peripheral blood lymphocytes of two donors (F1 and F3) in order to detect the primary DNA damage induced by hispidin in dose range of 25-1000 µM. In this study, comet parameters such as tail length (TL, µm), % of the DNA in tail (%TDNA), and tail moment (TM, the product of relative tail intensity and length) were evaluated, but only the data on the %TDNA are presented. %TDNA was a parameter of choice, because it is considered to be the most reliable as it covers the widest range of damage and is linearly related to DNA break frequency (Collins et al., 2008). Comparable results were obtained when tail length was analysed (data not presented).

The extent of DNA damage as measured by comet assay is presented in Fig. 3.23. Hispidin did not induce statistically significant increase in the %TDNA in the lymphocytes of both donors. Moreover, significant decrease in DNA damage was observed in the lymphocytes of donor F1 after treatment with 200 and 500 µM hispidin doses. A positive control we used was hydrogen peroxide (H₂O₂, 20 µM), which induced significant increase in the %TDNA in both donors (19.76±1.06 and 21.36±2.53, in donors F1 and F3 respectively).

Hispidin had no influence on cell viability (data not presented), which was evaluated using Casy TT Cell Counter and Analyzer (INNOVATIS, Switzerland) where quantification of cell viability is based on the dye-free non- invasive electrical current exclusion principle.

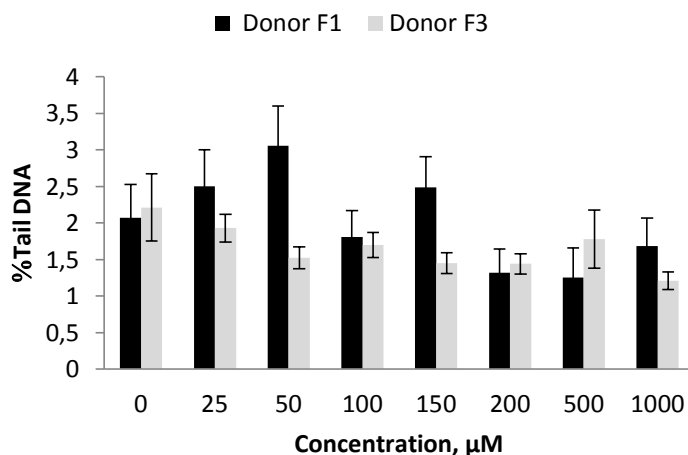


Fig. 3.28 Percentage of DNA in a comet tail (%TDNA± S.E.M.) after exposure of human lymphocytes *in vitro* with different concentrations of hispidin

3.7.2.2.4. *Salmonella*/microsome test

Ames test was carried out using Ames *Salmonella typhimurium* strains TA98 and TA100 with and without S9 metabolic activation (Table 3.9). All hispidin samples showed negative results in the Ames test, indicating that hispidin does not produce reverse mutation in bacterial cells.

Table 3.9 Mean number of revertants induced by fungal metabolite hispidin, the reference mutagen and the negative control in *Salmonella*/microsome test using TA98 and TA100 strains with or without S9 fraction

Treatment	Dose, µg/mL	TA 98 strain		TA100 strain	
		Revertant colonies/plate, mean±S.D.		Revertant colonies/plate, mean±S.D.	
		Without S9 fraction	With S9 fraction	Without S9 fraction	With S9 fraction
Blank control		51.50±13.44	26.50±2.10	80.50±9.19	64.00±7.07
Vehicle control		50.50±2.12	29.50±0.71	81.50±3.54	72.50±2.12
Daunomicine		813.00±24.04	–	–	–
Sodium Azide		–	–	498.50±58.69	–
2-Amino-anthracene		–	926.00±265.87	–	1622.50±204.35
Hispidin	50	42.67±5.03	34.67±3.06	97.33±6.34	82.00±18.19
	100	39.00±3.61	34.33±6.43	77.67±12.92	102.33±15.50
	150	40.67±3.06	33.00±3.61	92.33±9.84	83.33±19.86
	250	39.33±5.51	33.00±4.00	98.00±6.68	72.00±7.94

3.7.2.3. Determination of anthelmintic effect on gastrointestinal nematodes

Parasitic nematodes of the digestive tract are one of the most pathogenic parasites of ruminants. Adult worms attach to digestive system of goats or sheep are feed on the blood have serious consequences in warm humid climates. The most important limiting factor of infected ruminants is productivity and also health problems, which cause anemia, oedema and death (Joshi et al., 2011, Hoste et al., 2005). The typical treatment or prevention of infection of gastrointestinal nematodes depends on the repeated use of anthelmintics. However their continued use has led to anthelmintics resistance in worm populations and the presence of synthetic chemical residues in meat and milk have created markets for organically produced animals raised without synthetic anthelmintics (Jackson and Coop, 2000, Acharya, Hildreth and Reese, 2014). These aspects have induced scientist to search of efficient and alternative “natural” anthelmintics from plant origin that can be used in livestock production (Waller and Thamsborg, 2004).

To sustain a concept and pay attention on mushrooms, which are rich source of bioactive molecules with enormous variety of chemical structures such as phenolic compounds, polyketides, terpenes and steroids (Ferreira et al., 2009). Either some of (*Polyporus*) *Phaeolus* mushroom species were used in medicine as effective vermifuge in anthelmintic therapy (Lihua et al., 2010).

The isolated compounds of *P. schweiniztii* were tested as athelmintics agents on predominant hematophagous parasite *H. contortus* and often co-dominat in parasite populations *T. colubriformis*, although it could be as harmful as *Heamonchus* (Mahieu et al., 2007). The larval exsheathment assay has been developed to measure the ability of hispidin, and hispolon to delay or inhibit and artificial induced exsheathment process of infective L₃ larvae (Bahuaud et al., 2006). By preventing exsheathment (*Fig3.29*) process compounds possess anthelmintics activity and hereby larvae may not able to infect the animal; thus ensheathed (*Fig. 3.30*) larvae are excretive from digestive tract of animals (Hertzberg et al., 2002).



Fig 3.29 Exsheathed L₃ larvae of *T. colubriformis*

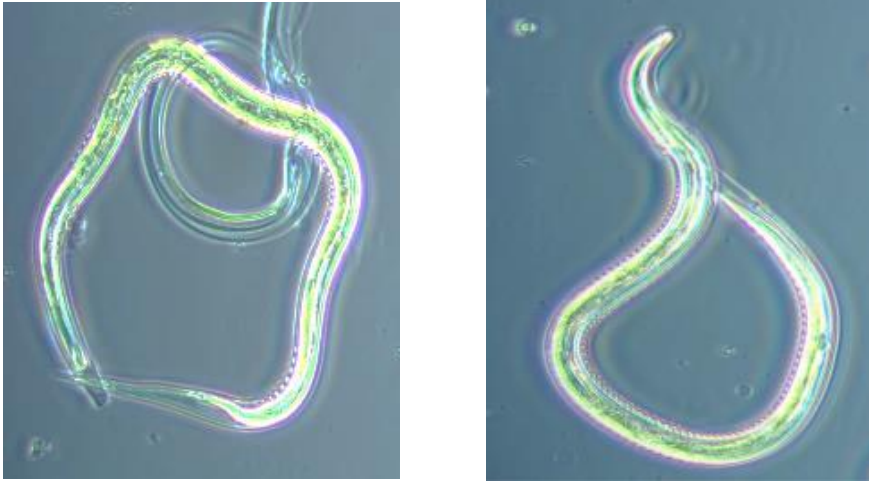


Fig. 3.30 Ensheathed L₃ larvae of *T. colubriformis*

In controls from 97-100 % of the *H. contortus* and *T. colubriformis* larvae were generally exsheathed after 60-minute contact with the solution for exsheathment (Fig. 3.31, 3.32 and 3.33.). Hispolon were not tested on *H. contortus* larvae, but provoked a slightly delay with concentration 300 µg/ mL in the exsheathment rate of *T. colubriformis* (Fig. 3.31). In 3-hour contact hispidin led to a strong inhibition of the both nematodes species with highest tested concentration 300 µg/ mL.

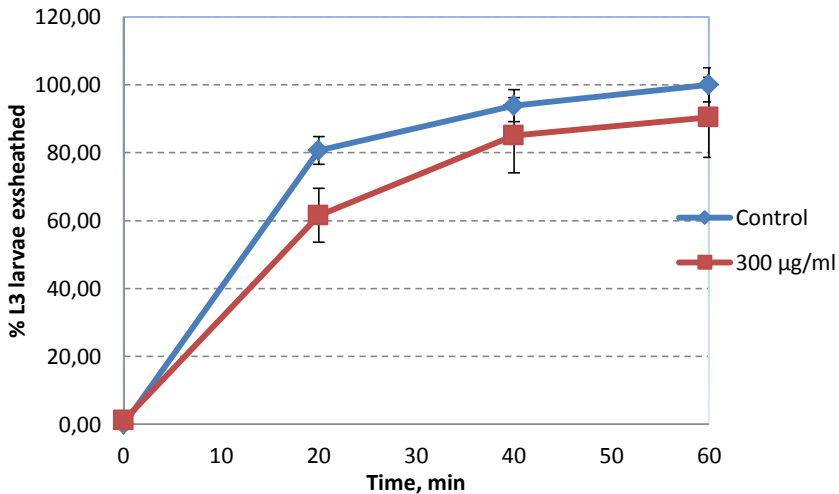


Fig. 3.31 Hispolon activity on *T. columbiformis* third-stage larvae on the process of artificial *in vitro* exsheathment.

The larvae were 3.97 ± 2.88 % and 16.52 ± 7.72 % exsheathed after 60 minutes for *T. colubriformis* and *H. contortus* respectively (Fig. 3.32 and 3.33). In the contrast, for the other concentration 150 and 75 $\mu\text{g}/\text{mL}$ exsheathment was significantly delayed (15-74% and 31-90% respectively, in the time 20-60 min.) for *T. colubriformis*. Contrary smaller concentration tested for *H. contortus* were less effective and the mean percentages ranged from 53% to 100% (150 $\mu\text{g}/\text{mL}$) and 84% to 100% (75 $\mu\text{g}/\text{mL}$) on the time process 60 min.

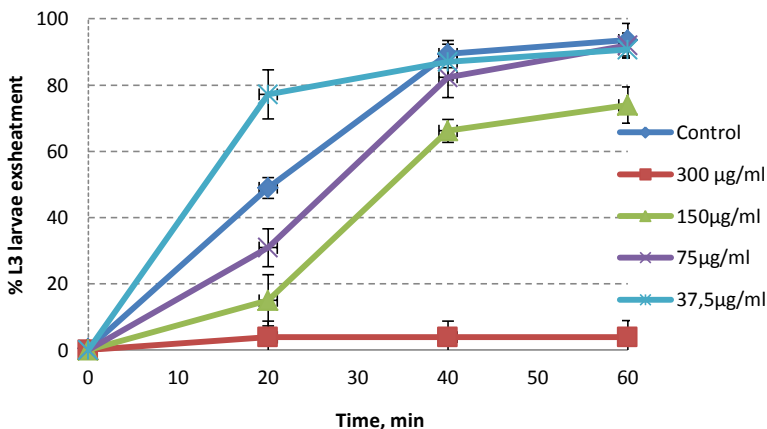


Fig. 3.32 Hispidin activity on *T. colubriformis* third-stage larvae on the process of artificial *in vitro* exsheathment

compounds may have influence the structural conformation and molecular weight of hispidin and hispolon. As the antelmintic effects of flavan-3-ols showed that the total inhibition of exsheathment was associated with the total number of hydroxyl groups, hence the molecular weight of the molecules, as well as with the presence of group in C ring (Brunet and Hoste, 2006).

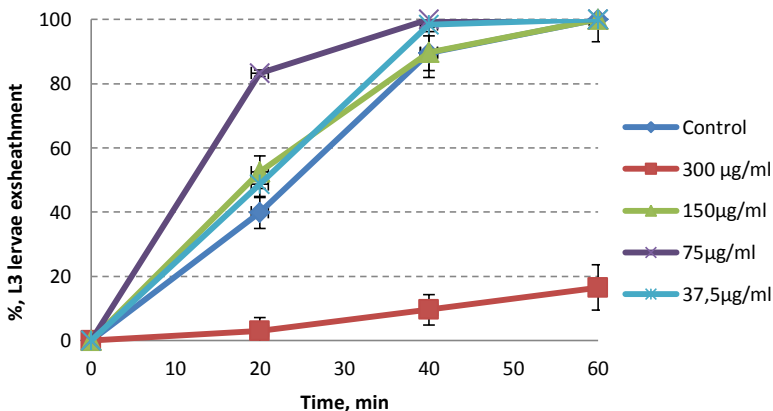


Fig. 3.33 Hispidin activity on *H. contortus* third-stage larvae on the process of artificial *in vitro* exsheathment

The changes in the exsheathment rate also belong on interaction between bioactive molecules and proteins composition of nematodes larvae. The polyphenols possibility to have antelmintic properties and affect biological process proved *in vitro* and *in vivo* assays. Several studies in sheep and goats describe the short term experimental design which did not permit the development and expression of larvae (Paolini et al., 2003a, Paolini et al., 2003b, Athanasiadou et al., 2001).

Unfortunately, the exact process of action remains undefined and could differ depending on the parasite strain, its stage of development, possible biochemical characteristics of the forage species and bioactive molecules structure (Hoste et al., 2006).

At the same time methanolic extract of *P. schweintzii* was tested against two nematodes, together with other methanolic extract of *I. hispidus* which contain isolated secondary metabolites described above. Both mushrooms were collected in course of two year (2011, 2012).

Exsheathment of *H. contortus* L₃ in the control groups was 97-100% of exsheathment obtained after 60 min. Compared to the control values, the strong inhibition of the larval exsheathment with the *I. hispidus* (2011) was at concentration 1200µg/mL and 600 µg/mL (Fig. 3.34). In contrast, although still significant the reduction in the exsheathment process was less pronounced at the 300 µg/mL and 150 µg/mL concentrations ineffective. Although *I. hispidus* (2012) of the larval potent inhibition possessed only with highest tested concentration (Fig.3.36), the 600 µg/mL exsheathment was significantly delayed, whereas other concentrations were less expressed.

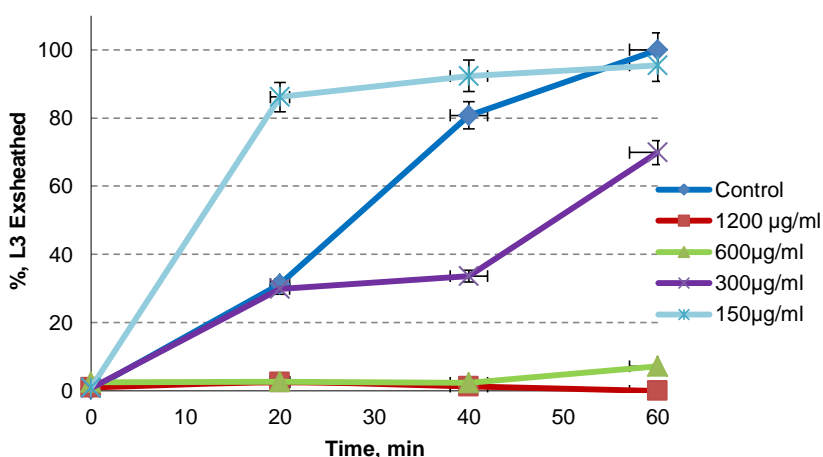


Fig. 3.34 Variations in the inhibition of the artificially induced exsheathment rate measured for *H. contortus* third stage larvae for control or after contact with *I. hispidus* (2011) extract

Compared to the control values, the contact with the extracts of *P. schweintzii* (2011) induced a highly significant, nearly total inhibition of the exsheathment process for the larvae observed at the concentrations of 1200, 600 and 300 µg/mL

(Fig. 3.35). Contrary *P.schweintzii* (2012) extract was ineffective and exsheathment process values were close to control values (Fig.3.37).

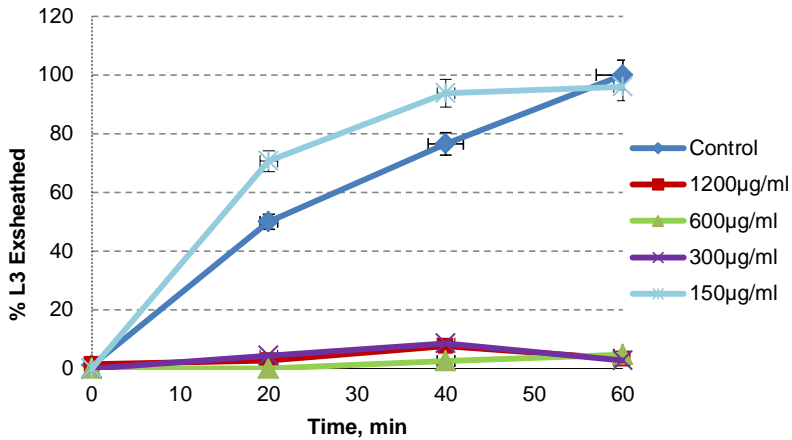


Fig. 3.35 Variations in the inhibition of the artificially induced exsheathment rate measured for *H. contortus* third stage larvae for control or after contact with *P. schweintzii* (2011) extract

After 60min, the exsheathment of *T. columbiformis* rates in controls ranged 92-100% for *P.schweintzii* and *I. hispidus*. Compared to the control values, the contact with the *I.hispidus* (2011) extracts induced a highly significant, nearly total inhibition of the larval exsheathment process at the concentrations of 600 and 1200 µg/mL, whereas lower concentration 300 µg/mL and was efficient and inhibited 41.40% ±4.16 larvae after 60min (Fig.3.38). Contrary the inhibition process was less efficient with *I.hispidus* (2012), highest concentration slightly inhibited larval exsheathment 67.80±4.49% in 60min (Fig. 3.40), meanwhile other concentrations were inactive.

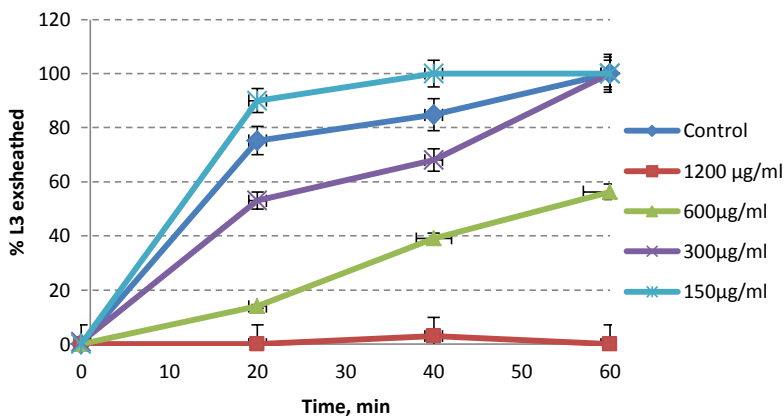


Fig. 3.36 Variations in the inhibition of the artificially induced exsheathment rate measured for *H. contortus* third stage larvae for control or after contact with *I. hispidus* (2012) extract

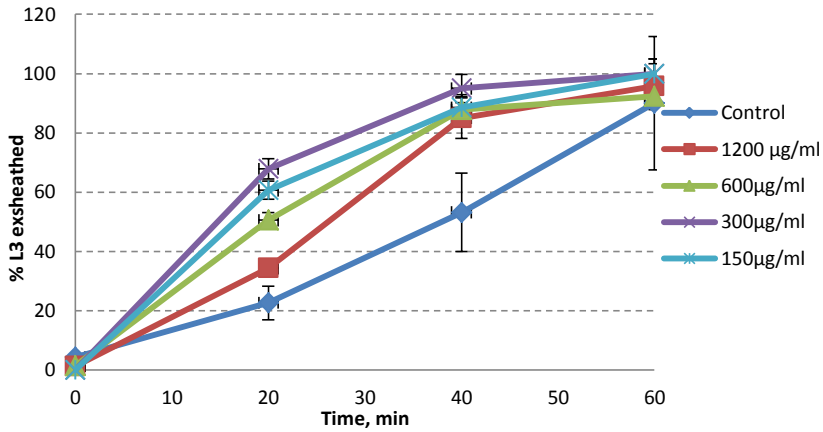


Fig. 3.37 Variations in the inhibition of the artificially induced exsheathment rate measured for *H. contortus* third stage larvae for control or after contact with *P. schweintzii* (2012) extract

With the *P. schweintzii* (2011) extracts, the inhibition of the larval exsheathment, compared to the control rates after 60 min, reached the highly efficient values of $2.76 \pm 2.64\%$ and $15.43 \pm 4.12\%$ at the concentrations of 1200, 600 g/mL (Fig. 3.39). However the difference between the rates observed after contact with the extracts at 300 and 150 µg/mL did not significantly differed after 60 min, but inhibition of 300 µg/mL was more efficient till 40min with value $26.19\% \pm 3.92$. Although *P. schweintzii* (2012) concentration 1200 µg/mL induced larval exsheathment $21.04 \pm 4.49\%$ in 60min (Fig.3.41), while other concentrations were inactive and showed similar activity as control.

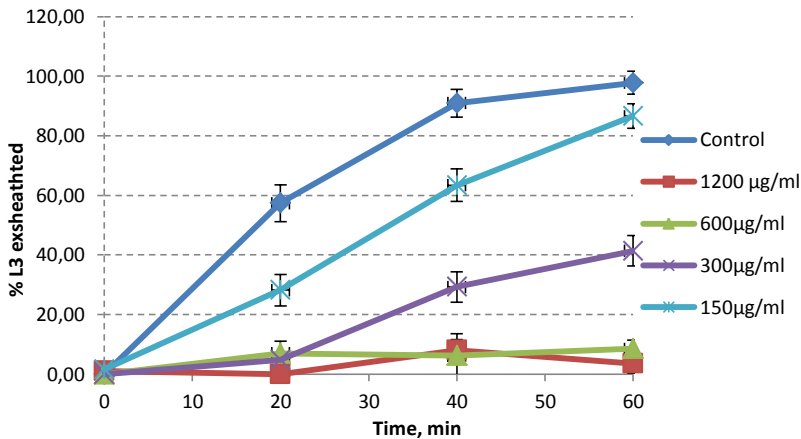


Fig. 3.38 Variations in the inhibition of the artificially induced exsheathment rate measured for *T. colubriformis* third stage larvae for control or after contact with *I. hispidus* (2011) extract

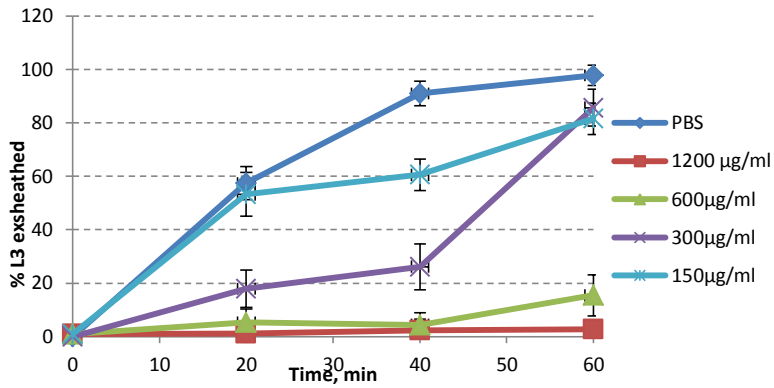


Fig. 3.39 Variations in the inhibition of the artificially induced exsheathment rate measured for *T. colubriformis* third stage larvae for control or after contact with *P. schweintzii* (2011) extract

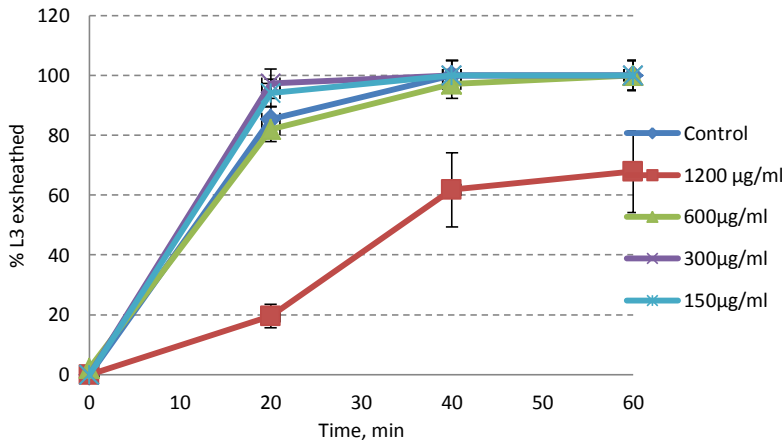


Fig. 3.40 Variations in the inhibition of the artificially induced exsheathment rate measured for *T. colubriformis* third stage larvae for control or after contact with *I. hispidus* (2012) extract

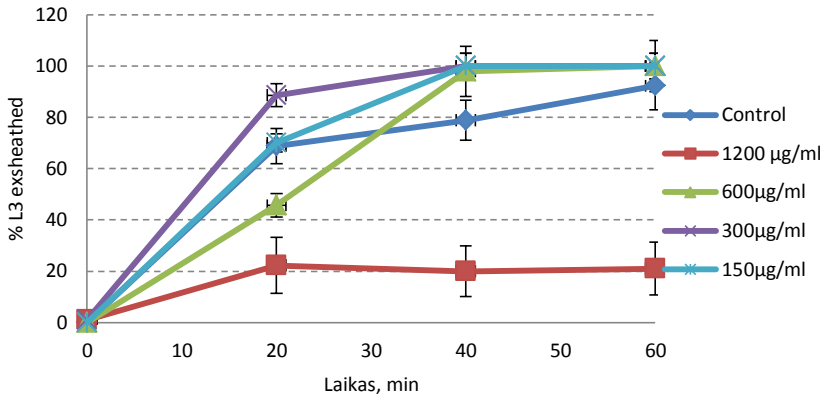


Fig. 3.41 Variations in the inhibition of the artificially induced exsheathment rate measured for *T. colubriformis* third stage larvae for control or after contact with *P. schweintzii* (2012) extract

The present results confirmed the antelmintic properties of both *P.schweintzii* and *I. hispidus*. The effects of both mushrooms were year dependent for both tested nematodes. Mushrooms collected in 2011 showed better activity, and were more effective with lower concentrations. For example *P.schweintzii* (2011) had strong inhibition activity (1200 and 600 µg/mL) and induced less than 20% of exsheathment during 60min on *H.contortus* and *T. colubriformis*, also similar results possessed *I. hispidus*. Less efficient results on two nematodes expressed *P.schweintzii* and *I. hispidus* collected in 2012, slightly delay demonstrated only highest concentration and inhibition exsheathment varied from 21-68% or smaller concentrations (150 and 300 µg/mL) were inactive. These differences may have several factors, such as bioactive components composition and concentration in mushrooms, collection period or age of the species. For instance, salt in soil modify flavonoid composition in plants (Tattini and Traversi, 2008).

The contact with the *P.schweintzii* (2011) extracts at the 3 concentrations tested, resulted in a nearly total inhibition of exsheathment, which was found with the *H.contortus*. The inhibitory effects observed with the *I. hispidus* extracts were less efficient and two concentrations showed slight delay. The extracts of *I.hispidus* (2011) *P.schweintzii* (2011) activity were more dependent of the year collection and showed potent efficiency two concentrations with *T. colubriformis*, thus highest concentration were slightly retain for 2012 year collection.

A possible explanation could be that the two nematode parasites occupy different locations in the animal, and also the activating conditions for exsheathment vary between abomasal and intestinal nematodes. However, from the results it is evident that extracts of *P. schweintzii* and *I. hispidus* caused insignificant delays of exsheathment in a dose dependent manner for both nematodes especially for 2011 year collection. The effect of well know forage legume sainfoin extract was tested of *H. contortus* and *T.colubriformis* L3 larvae at concentrations of 300, 600 and 1200µg/ mL. At the 1200µg/mL concentration, nearly total inhibition of exsheathment of *H. contortus* and *T. colubriformis* was observed (Brunet and Hoste, 2006). In our study, similar results were observed. Variability of exsheathment inhibition between mushroom species could be due to several reasons and may not necessarily reflect a true difference between nematode species (Novobilský, Mueller-Harvey and Thamsborg, 2011). The extracts probably differed in secondary metabolites contents and maybe have synergetic effect in the species between *P.schweintzii* and *I.hispidus*. Numerous research activities on gastrointestinal nematodes strains explains duo to flavonoids and, in particular tannins rich plants (Acharya et al., 2014, Manolaraki et al., 2010, de Oliveira et al., 2011, Katiki et al., 2011, Alonso-Díaz et al., 2008). However, besides polyphenols, some other classes of biochemical compounds can also participate in the bioactivity and inhibit parasites. This was found with chestnut or pine tree extracts when using the exsheathment inhibition assay (Azando et al., 2011). Isolation of compounds and precise activity identification is one of the factors to understand the biochemical pathways affected by exposure to the mushroom extracts. Such as isolated bioactive

molecules (hispidin, hispolon) from *P. schweintzii* inhibit exsheathment on gastrointestinal nematodes and possessed anthelmintic properties.

In the last decade drug discovery programs for new anthelmintic compounds are expensive and have produced relatively few new compounds (Csermely et al., 2013, Mackenzie and Geary, 2013). Recently important implications for the potential therapies occur that cocktails of plant extracts combined with existing anthelmintic drugs may overcome some of the emerging drug resistance problems for some commercially available drugs (Kumarasingha et al., 2014). Therefore, it is important to concentrate on natural matter and find novel drug with anthelmintic properties, not only by replace the existing drug, but also to prolong the effective life cycle of the existing drugs.

In all tested antioxidant assays (RSC, ORAC and FRAP) hispidin, with catechol moiety exhibited similar or sometimes more potent activity than Trolox.

Hispidin, hispolon and inonic acid methyl ester demonstrate moderate activity against on promastigotes - *L. infantum*.

The detailed biological activity *in vitro* of hispidin on human lymphocytes in the micronucleus and induction of chromosome aberrations in human lymphocytes did not induced genotoxicity. Either, hispidin did not induced primary DNA damage and mutation in bacterial cell.

Isolated compounds showed anthelmintic activity against *H. contortus* and *T. colubriformis* gastrointestinal nematodes with strongest tested concentration (300 µg/mL). Compounds activity of gastrointestinal nematodes may determine the structural conformation and molecular weight of hispidin and hispolon.

Either extracts of *P. schweintzii* and *I. hispidin* were potent inhibitors on larvae exsheathment. The differences between mushroom specie may occur due to secondary metabolites content and synergetic effect of molecules in *P. schweintzii* and *I. hispidus*.

4. CONCLUSIONS

1. Sequential fractionation with increasing polarity and dielectric constant of solvents was applied in our study. The highest polarity water and methanol enabled to obtain the highest extracts yields from 2.94 (*H. ferrugineum*) -25.29 % (*T. caligatum*) and from 6.77 % (*S. halophila*)-41.29 % (*A. pseudopratisensis*), respectively. The yields of cyclohexane and dichloromethane extracts were remarkably lower from 0.61(*S. halophila*)-5.14% (*R.badia*), 0.52(*G.ammophilus*)-3.42 % (*T. caligatum*), respectively. Dominated families possessed significant total yields were Agaricaceae, Tricholomataceae, Boletacea.

2. The following factors had effects on antioxidant capacity of mushroom extracts measured by the *in vitro* ABTS⁺⁺, DPPH[·], FRAP, ORAC and TPC assays: mushroom family and species, solvent type and polarity, evaluation method. The highest 'antioxidant scores', which were introduced for integrating numerous antioxidant evaluation data and expressed in comparative integrated units per g of mushroom dry weight, demonstrated *I. hispidus* (808 $\mu\text{g}/\text{g}$ mdw), *P. schweinitzii* (712 $\mu\text{g}/\text{g}$ mdw) and *C. infractus* (481 $\mu\text{g}/\text{g}$ mdw). In general, the species of Agaricaceae and Boletaceae families possessed superior antioxidant potential, which was in the ranges of 109-325 and 140-253 $\mu\text{g}/\text{g}$ mdw, respectively. The correlation between total phenolics and total antioxidant activity observed in our study was not as strong as may be expected, most likely due to the presence of various compound classes participating in the applied assays by different mechanisms. In general, the extracts isolated with polar solvents were characterised as possessing higher antioxidant activity.

3. The majority of mushroom extracts (89% of the total samples tested) demonstrated antimicrobial activity, 59% of them characterize as partial antimicrobial activity and not fully inhibiting microorganisms. Methanol fractions of *P. schweinitzii*, *I. hispidus* and *C. infractus* were the strongest antimicrobial agents against tested microorganisms in disc diffusion assay (inhibition zones were from 10 to >15 mm) and bioautography method. It should be noted that the same species demonstrated the highest antioxidant potential Gram-positive bacteria, *Bacillus cereus* and *Staphylococcus aureus* were more sensitive to mushroom extracts than Gram-negative, *Pseudomonas aeruginosa* and *Escherichia coli*.

4. Cyclohexane extracts of *G. ammophilus* as well as cyclohexane and dichloromethane extracts of *S. halophila* exhibited good cytotoxic activity against parasite *Leishmania infantum* amastigotes: the selectivity indexes (SI) indicating ratio between, cytotoxicity and activity against parasite for the above-mentioned extracts were 49.09, 22.28, and 22.42, respectively. These findings are promising for the further development of novel natural antiparasite preparations.

5. *P. schweinitzii* methanolic extract, as the substance possessing strong antioxidant capacity and antimicrobial activity, was fractionated by thin layer

(TLC), column (CC) and high performance liquid chromatography (HPLC): TLC-DPPH[•] bioautography assay revealed 2 fractions exhibiting remarkable radical scavenging capacities which were purified by CC and HPLC.

6. Three principal constituents, namely hispidin, hispolon and inonotic acid methyl ester were elucidated and identified in *P. schweinitzii* by using a range of spectroscopic techniques - ESI-MS, ESI-MS/MS, ¹H NMR, ¹³C NMR, HMBC, COSY, HSQC. Hispolon and inonotic acid methyl ester are reported in *P. schweinitzii* for the first time.

7. The activity of the purified principal components were tested for their antioxidant capacity and cytotoxicity. Hispidin was an effective antioxidant in ABTS^{•+}, DPPH[•], FRAP, ORAC assays; in general, its activity was similar to the strong synthetic antioxidant, Trolox. The *in vitro* assessment of hispidin on human lymphocytes in the micronucleus and induction of chromosome aberrations in human lymphocytes did not reveal its genotoxicity. Either, hispidin did not induced primary DNA damage and mutation in bacterial cell. Hispidin, hispolon and inonotic acid methyl ester demonstrated an activity against promastigotes *L. infantum* and had immunomodulatory effects modifying the antiparasitic response of infected macrophages.

8. The extracts of *P. schweintzii* and *I. hispidus* were potent inhibitors on larvae exsheathment against *H. contortus* and *T. colubriformis* gastrointestinal nematodes; however, the effects of extracts isolated from the mushrooms collected in different years were different, indicating that active components may be present at different concentrations. Hispidin led to a strong inhibition with highest tested concentration (300 µg/ mL) against both tested nematodes, while hispolon provoked a slightly delay against *T. colubriformis*.

9. In general, comprehensive and systematic evaluation of antioxidant and antimicrobial properties of underinvestigated wild mushroom species provides an important data for their further valorisation as a source of valuable ingredients. More specifically, the extracts isolated from the species possessing the strongest antioxidant potential may be promising protecting agents against oxidative damage and inhibitors of gastrointestinal nematodes. The principal components of such species, hispidin, hispolon and inonotic acid methyl ester may represent a group of new natural compounds for developing functional ingredients for nutraceuticals, functional foods, pharmaceuticals and other applications. Further research should be focused on elucidating the mechanisms of performance of extracts, fractions and purified compounds using wider spectra of the *in vitro* (e.g. cell cultures) and *in vivo* studies.

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APPENDIX 1

Sorted families and the number of active species against tested bacteria

Family	fraction	Number of species	Number of active species against Gram positive bacteria		Number of active species against Gram negative bacteria	
			<i>S. aureus</i>	<i>B. cereus</i>	<i>P. aeruginosa</i>	<i>E.coli</i>
<i>Agaricaceae</i>	C	7	2	3	2	0
	D	7	7	5	4	5
	M	9	6	6	5	4
<i>Tricholomataceae</i>	C	5	3	2	2	2
	D	5	4	2	3	2
	M	6	4	5	5	2
<i>Boletaceae</i>	C	5	3	3	4	1
	D	3	2	1	2	2
	M	5	3	3	4	3
<i>Cortinariaceae</i>	C	2	0	1	1	1
	D	1	1	1	1	0
	M	2	1	1	1	2
<i>Russulaceae</i>	C	2	0	0	0	0
	D	2	0	0	1	0
	M	2	2	1	2	2
<i>Gyroporaceae</i>	C	1	1	1	0	0
	D	1	1	1	1	1
	M	1	1	1	1	0
<i>Strophariaceae</i>	C	1	0	0	0	1
	D	1	1	0	0	0
	M	1	1	1	1	0
<i>Amanitaceae</i>	C	1	0	0	1	0
	D	1	0	0	1	1
	M	1	1	1	1	0
<i>Polyporaceae</i>	C	2	0	2	2	0
	D	2	0	2	2	0
	M	2	0	2	2	0
<i>Bankeraceae</i>	D	1	0	1	0	0
	M	1	0	1	1	0
	Total		80	44	47	50

C, cyclohexane; D, dichloromethane; M, methanol;

SCIENTIFIC PUBLICATIONS

„ISI Web of Science“

Smolskaitė, Lina; Venskutonis, Petras Rimantas; Talou, Thierry. Comprehensive evaluation of antioxidant and antimicrobial properties of different mushroom species. *LWT-Food Science and Technology*, 2015, 60.1: 462-471.

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Comprehensive evaluation of antioxidant and antimicrobial properties of different mushroom species



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ARTICLE INFO

Article history:

Received 16 December 2013

Received in revised form

16 April 2014

Accepted 10 August 2014

Available online 17 August 2014

Keywords:

Mushrooms

Extracts

Antioxidant activity

Antimicrobial activity

Antioxidant score

ABSTRACT

Antioxidant properties of mushroom extracts sequentially isolated by cyclohexane, dichloromethane, methanol, and water from *Phaeolus schweinitzii*, *Inonotus hispidus*, *Tricholoma columbetta*, *Tricholoma caligatum*, *Xerocomus chrysenteron*, *Hydnellum ferrugineum*, *Agaricus bisporus* and *Pleurotus ostreatus* were evaluated by DPPH•, ABTS^{•+} scavenging capacity, ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC), and Folin–Ciocalteu total phenolic content (TPC) methods. The integrated values ('antioxidant scores') for evaluating antioxidant potential of extracts and dry mushroom substances are proposed. Antimicrobial activity was screened against Gram-positive (*Bacillus cereus*), Gram-negative (*Pseudomonas aeruginosa*) bacteria and fungi (*Candida albicans*) by agar diffusion method. The highest antioxidant capacity values (in $\mu\text{M TE/g}$ extract dw) were found for methanol fractions of *P. schweinitzii* (9.62 ± 0.03 in DPPH•; 109 ± 3 in FRAP; 164 ± 1 in ABTS^{•+}; 340 ± 3 in ORAC assays) and *I. hispidus* (9.5 ± 0.04 in DPPH•; 54.27 ± 0.46 in ABTS^{•+}; 88.31 ± 1.96 in FRAP; 290 ± 1 in ORAC assays). Extracts of other species possessed considerably lower antioxidant activities. The extracts of *I. hispidus* were more effective against tested microbial species than other mushrooms. In conclusion, our results show that some wild mushrooms might be promising dietary sources of natural antioxidants and antimicrobial agents.

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1. Introduction

The role of free radicals in the development of various diseases is thoroughly discussed. Deficiency in endogenous antioxidant defense may result in oxidative stress, which might be associated with various health problems, including coronary heart diseases, neural disorders, diabetes, arthritis and cancer (Yoshikawa, Toyokuni, Yamamoto, & Naito, 2000, chap. 2). Therefore, dietary antioxidants are believed to assist in maintaining good health, as well as in preventing various diseases (Augustyniak et al., 2010). Antioxidants are present in all biological systems; however, plant kingdom remains the main source of healthy compounds. Therefore, search for effective and non-toxic natural antioxidants and other bioactive molecules have become a regularly increasing topic. In addition, many phytochemicals possess antimicrobial activity, which can also be applied for food and medical purposes.

Mushrooms have been widely used as a human food for centuries and have been appreciated for texture and flavour as well as

various medicinal and tonic properties. However, the awareness of mushrooms as being an important source of biological active substances with medicinal value has only recently emerged. A number of mushroom species has been reported during last decade to possess significant antioxidant activity (Hearst et al., 2009; Jones & Janardhanan, 2000; Kalogeropoulos, Yanni, Koutrotsios, & Aloupi, 2013; Mathew, Sudheesh, Rony, Smina, & Janardhanan, 2008; Nitha, Strayo, Adhikari, Devasagayam, & Janardhanan, 2010). Mushrooms are also rich in proteins, fiber, vitamins and minerals, while the content of fat is low (Guillamón et al., 2010). In addition, edible mushrooms usually contain various bioactive molecules, such as phenolic compounds, polyketides, terpenes and steroids (Barros, Baptista, & Ferreira, 2007). Mushrooms played an important role in the treatment of various disorders, including infectious diseases and therefore some naturally occurring chemical compounds identified in mushrooms served as models for clinically proven drugs (Barros, Cruz, Baptista, Estevinho, & Ferreira, 2008). However, the interest in the use of mushrooms for the development of nutraceuticals and functional food ingredients is quite recent. In fact, due to multipurpose applications and uses mushrooms should be considered not only as a traditional food but also as a source of high value flavourings, efficient natural dyes, as well as a raw

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material for functional food, food supplement and pharmaceutical ingredients.

The aim of this study was to apply biorefinery approach in order to valorise some wild mushrooms growing in Midi-Pyrénées region, which are further briefly reviewed. *Tricholoma columbetta* is edible and can be consumed fresh, dry or pickled. A cyclopentene derivative columbetdione (Vadalà, Finzi, Zanoni, & Vidari, 2003) and endopeptidase (Lamaison, Pourrat, & Pourrat, 1980) were found in its fruiting bodies, while ethyl acetate extracts of *T. columbetta* were shown to possess nematocidal activity against *Caenorhabditis elegans* and antibacterial activity against *Bacillus brevis* (Stadler & Sterner, 1998).

Phaeolus schweinitzii (Fr.) is a common root and butt pathogen of conifers in North America and Eurasia producing a strong, water-soluble pigments possessing five intensive colours: olive-brown, olive-grey, dark-brown, brownish-grey and linoleum-brown (Cedano, Villaseñor, & Guzmán-Dávalos, 2001). Hispidin was isolated from acetone extract of *P. schweinitzii* (Ueno, Fukushima, Saiki, & Harada, 1964). *Inonotus hispidus* is a parasitic fungus preferably living on deciduous trees such as *Fraxinus*, *Quercus*, *Sorbus* and *Malus*. It has been used as a traditional medicine for treating dyspepsia, cancer, diabetes and stomach problems in the northeast region and Xinjiang province of China (Ali, Jansen, Pilgrim, Liberra, & Lindequist, 1996). *I. hispidus* contains polyphenol pigments with styrylpyrone skeleton, which were reported to exhibit antimicrobial, antioxidant, antiviral and anti-inflammatory activities. Two natural antioxidants, named inonotusin and hispidin were isolated from the methanolic extract of the fruit bodies and showed antioxidant and cytotoxic activity against human breast carcinoma cells (Zan et al., 2011).

Xerocomus chrysenteron is an edible mushroom occasionally harvested in autumn. The lectin was identified in *X. chrysenteron* (Birck et al., 2004), while its methanolic extract was reported to possess antioxidant activity (Heleno et al., 2012; Sarikurkcu, Tepe, & Yamac, 2008). *Tricholoma caligatum* forms a small and fuscous to blackish fruit body and grows in the Mediterranean region (Murata, Ota, Yamada, Yamanaka & Neda, 2013). Various aromatic derivatives were identified in *T. caligatum* (Fons, Rapior, Fruchier, Saviuc, & Bessière, 2006).

Agaricus bisporus (the button mushroom) is the most widely cultivated form in the USA, Europe and different parts of Australasia. It is recognized as a source of unsaturated fatty acids such as linoleic, linolenic, conjugated linoleic and polyphenols (Singh, Langowski, Wanib & Saengerlauba, 2010) demonstrating some medicinal properties such as anticancer activity (Shi, James, Benzie, & Buswell, 2002; Zhang, Huang, Xie & Holman, 2009). *Hydrellium ferrugineum* has red spore deposit and is easily recognized. *Hydrellium* spp. are regarded as “nitrogen sensitive” organisms (Ainsworth, Parfitt, Rogers, & Boddy, 2010; Van der Linde, Alexander, & Anderson, 2008) and have become a concern of European conservation. *Pleurotus ostreatus* is a highly nutritious edible mushroom and is considered as a source of valuable nutritional and medicinal compounds; it can be easily cultivated on a large range of substrates (Gern, Wisbeck, Rampinelli, Ninow, & Furlan, 2008). It could be used as a cholesterol lowering additive in human diet (Schneider et al., 2011).

Regardless above-cited articles, the reports on the antioxidant activity and antimicrobial properties of the selected in this study mushrooms are rather scarce; previously published data is particularly lacking a systematic approach and comprehensiveness. Therefore, the aim of the present work was to evaluate antioxidant and antimicrobial properties of the selected mushroom species by applying a more systematic approach. The antioxidant potential was comprehensively evaluated for mushroom fractions sequentially isolated with cyclohexane, dichloromethane, methanol, and water by using free radical scavenging capacity, oxygen radical

absorbance capacity, ferric reducing antioxidant capacity and total phenolics content assays. Antimicrobial activity was screened against Gram-positive (*Bacillus cereus*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria and fungi (*Candida albicans*).

2. Materials and methods

2.1. Mushrooms and chemicals

Phaeolus schweinitzii, *Inonotus hispidus*, *Tricholoma columbetta*, *Tricholoma caligatum*, *Xerocomus chrysenteron*, *Hydrellium ferrugineum* were harvested in Midi-Pyrénées region of France in autumn 2009. Taxonomic identification was carried out by Mycologist Association of Faculty of Pharmacy of Toulouse University. *Agaricus bisporus* and *Pleurotus ostreatus* were purchased in the local supermarket. All freeze-dried (Lyophilisateur pilote LPCPLS15, Cryotec, Saint-Gély-du-Fesc, France) mushrooms were ground in a Microfine mill (MF-10, IKA, Staufen, Germany) through a 1.5-mm sieve and then stored in air-tight plastic bags in a desiccator at room temperature for further analysis.

Stable 2,2-di-phenyl-1-picrylhydrazyl hydrate radical (DPPH•, 95%), gallic acid, anhydrous sodium carbonate, 6-hydroxy-2-5-7-8-tetramethylchroman-2-carboxylic acid (Trolox, 97%), 2,20-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), fluorescein (FL) and [2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH)] were from Sigma–Aldrich (Steinheim, Germany); 2.0 M Folin–Ciocalteu phenol reagent, KCl, NaCl, Na₂HPO₄, Na₂CO₃ and K₂S₂O₈ were from Merck (Darmstadt, Germany); KH₂PO₄ from Jansen Chimica (Beerse, Belgium); methanol, 98% acetic acid from Lachema (Brno, Czech Republic) and agricultural origin ethanol (96.6%) from Stumbras (Kaunas, Lithuania); 2,4,6-tripyridyl-s-triazine (TPTZ) was from Fluka Chemicals (Steinheim, Switzerland).

2.2. Extraction procedure

HPLC grade cyclohexane, dichloromethane, methanol and deionized water were used to fractionate soluble compounds from the mushrooms in ascending polarity by sequentially extracting 2–100 g (depending on material availability) ground mushrooms in a Soxhlet extractor for 5 h. The samples were air dried after each solvent extraction and finally the residues were extracted with boiling water during 5 h constantly mixing in the Ikamag “RTC basic” magnetic stirrer (IKA Labortechnik, Staufen, Germany). Organic solvents were removed in a vacuum rotary evaporator RV 10 (IKA, Staufen, Germany), while water extracts were freeze-dried. All extracts were kept in a refrigerator until further analysis.

2.3. Antioxidant activity assays

2.3.1. DPPH•-scavenging capacity

This method is based on scavenging DPPH• by the antioxidant (Brand-Williams, Cuvelier, & Berset, 1995). The assay was performed in a 96-well microtiter plates using an UV spectrophotometer EL × 808 Microplate Reader (BioTex Instruments, Vermont, USA). The reaction mixture in each of the 96-wells consisted of 7.5 µL of different concentration mushroom extracts (0.5%; 0.25%; 0.125%) and 300 µL of methanolic solution of DPPH• (6×10^{-5} M). The mixture was left to stand for 40 min in the dark and the reduction of DPPH• was determined by measuring the absorption at 515 nm. All measurements were performed in triplicate. Radical scavenging capacity (RSC) was determined from the calibration curve, which was drawn by using 50, 100, 125, 250, 500, 1000 µM/L concentration solutions of Trolox and expressed in µM of Trolox equivalents (TE) per g dry extract weight (µM TE/g edw).

2.3.2. ABTS^{•+} decolourisation assay

The Trolox equivalent antioxidant capacity (TEAC) assay is based on the scavenging of ABTS^{•+} by the antioxidant which may be measured spectrophotometrically (Re et al., 1999). A stock solution of 2 mM ABTS was prepared by dissolving reagent in 50 mL of phosphate buffered saline (PBS) obtained by dissolving 8.18 g NaCl, 0.27 g KH₂PO₄, 1.42 g Na₂HPO₄ and 0.15 g KCl in 1 L of Milli-Q water. If pH was lower than 7.4, it was adjusted with NaOH. ABTS^{•+} was produced by reacting 50 mL of ABTS stock solution with 200 μL of 70 mM K₂S₂O₈ solution in purified water and allowing the mixture to stand in the dark at room temperature for 15–16 h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. For the assessment of extracts, the ABTS^{•+} solution was diluted with PBS to obtain the absorbance of 0.800 ± 0.030 at 734 nm. One mL of ABTS^{•+} solution was mixed with 10 μL extract solution in 96-well microtiter plates. The absorbance was read at ambient temperature every minute during 40 min. PBS solution was used as a blank; all measurements were performed in triplicate. The TEAC was determined from the calibration curve, which was drawn using 50, 100, 125, 250, 500, 1000 μM/L concentration solutions of Trolox and calculated in μM TE/g edw as follows:

$$\text{TEAC} \left(\frac{\mu\text{M}}{\text{g}} \right) = \frac{\text{TE}_s}{1000} \times \frac{V_s}{m_s}, \quad \text{TE}_s - \text{antioxidant activity of sample expressed in TE} (\mu\text{M}), V_s - \text{sample volume (mL)}, m_s - \text{sample mass (g)}.$$

2.3.3. Ferric-reducing antioxidant power (FRAP) assay

FRAP assay is based on the reduction of Fe³⁺ in its tripyridyl-triazine complex to the blue Fe²⁺ form (Benzie & Strain, 1999). The final results were expressed in μM TE/g edw. The FRAP reagent was prepared from acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily and was warmed to 37 °C in a water bath prior to use. Ten μL of sample were added to 300 μL of the FRAP reagent and 30 μL water. The absorbance of the reaction mixture was then recorded at 593 nm after 4 min. All measurements were performed in triplicate. The TEAC values were determined as indicated in previous sections.

2.3.4. Oxygen radical absorbance capacity (ORAC)

ORAC method was performed as described by Prior, Wu, and Schaich (2005) and Dávalos, Gómez-Cordovés, and Bartolomé (2004) by using fluorescein as a fluorescent probe. The reaction was carried out in a 75 mM phosphate buffer (pH 7.4); stock solution of fluorescein was prepared according to Prior et al. (2005). Mushroom extracts were diluted 1:1000 (w/v); 25 μL of extract and 150 μL of fluorescein (14 μM) solutions were placed in 96 transparent flat-bottom microplate wells, the mixture was preincubated for 15 min at 37 °C and 26 μL of AAPH solution (240 mM) as a peroxy radical generator added with a multichannel pipette. The microplate was immediately placed in the FLUORstar Omega reader (BMG LABTECH, Ortenberg, Germany), automatically shaken prior to each reading and the fluorescence was recorded every cycle (66 s), totally 150 cycles. The 485-P excitation and 520-P emission filters were used. At least 4 independent measurements were performed for each sample. Raw data were exported from the Mars software to an Excel 2003 (Microsoft, Roselle, IL) sheet for further calculations. Antioxidant curves (fluorescence versus time) were first normalized and from the normalized curves the area under the fluorescence decay curve (AUC) was calculated as $\text{AUC} = 1 + \sum_{i=1}^{i=80} \frac{f_i}{f_0}$, where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i . The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area under the curve (AUC). The TEAC values were determined as described in previous sections.

2.3.5. Determination of total phenolic content (TPC)

The TPC was measured with Folin–Ciocalteu reagent as originally described by Singleton, Orthofer, and Lamuela-Raventos (1999). Briefly, 30 μL (0.1%) of sample were mixed with 150 μL of 10-fold diluted (v/v) Folin–Ciocalteu reagent, and 120 μL of 7.5% Na₂CO₃. After mixing of all reagents, the microplate was placed in the reader and shaken for 30 s. After incubation for 30 min at room temperature the absorbance of the mixtures was measured at 765 nm. All measurements were performed in triplicate. A series of gallic acid solutions in the concentration range of 0.025–0.35 mg/mL was used for the calibration curve. The results were expressed in mg of gallic acid equivalents per g of dry extract weight (mg GAE/g edw).

2.4. Antibacterial assay

The antimicrobial activity was assessed by the disk-diffusion method (Bauer, Kirby, Sherris & Turck, 1996). The bacterial cell suspension was prepared from 24 h culture and adjusted to an inoculation of 1×10^6 colony forming units per mL (cfu/mL). Sterile nutrient agar (Bit Phar. acc EN 12780:2002, 28 g/L distilled water, Scharlau, Barcelona, Spain) was inoculated with bacterial cells (200 μL of bacterial cell suspension in 20 mL medium) and poured into dishes to obtain a solid plate. Twenty mg of test material dissolved in the same solvent of the extraction were applied on sterile 5 mm diameter paper discs, which were deposited on the surface of inoculated agar plates. The plates with bacteria were incubated for 24 h at 37 °C. Inhibition zone diameters around each of the disc (diameter of inhibition zone plus diameter of the disc) were measured and recorded at the end of the incubation time. An average zone of inhibition was calculated from 3 replicates. Paper discs with solvents were used as controls.

Minimal inhibitory concentrations (MIC) showing the lowest concentration of extract able to inhibit any visible microbial growth was determined by the agar diffusion technique (Rajbhandari & Schöpke, 1999). The highest concentration of extract tested during the experiment was 20 mg/mL. The extracts were prepared at the series of concentrations (0.01; 0.1; 1; 10; 20 mg/mL). Ten μL of each concentration solution was transferred in the disk. Then the disks were transferred in the Petri dishes containing microorganism culture. The plates were incubated for 24 h at 37 °C for bacteria. After incubation, the number of colonies in each plate was counted. Each assay replicated three times.

2.5. Statistical analysis and data assessment

The values are expressed as a means of 3 replicate measurements in antimicrobial assay and 4 replicates in antioxidant assays with standard deviations (SD). Correlation coefficients (R) to determine the relationship between two variables, RSC, FRAP, ORAC and TEAC tests were calculated using MS Excel 2010 software (CORREL statistical function). The antioxidant characteristics were summarized by using integrated values for extracts – ‘antioxidant score’ of extract (ASE), which is the sum of values for the fraction obtained with the same solvent in all assays, expressed in the so-called ‘comparative integrated units’ in g of dry extract weight (ciu/g edw) and for the whole plant dry material, expressed in g of dry mushroom weight (ciu/g mdw). The latter values, which may be called ‘antioxidant scores of mushrooms’ (ASM) take into the account ASE and extract yields (EY) and were calculated as follows: $\text{ASE}_c \times \text{EY}_c/100 + \text{ASE}_d \times \text{EY}_d/100 + \text{ASE}_m \times \text{EY}_m/100 + \text{ASE}_w \times \text{EY}_w/100$. These integrated values to some extent reflect the overall total antioxidant potential of different mushroom species, which also consider extract yields, as well as the effectiveness of different polarity solvents used for the extraction. It is

expected that the concept of antioxidant scores may help in assessing a large number of antioxidant activity data, which were obtained in this study.

3. Results and discussion

3.1. Extraction yield

Efficient extraction of antioxidants and other biologically active molecules requires the use of solvents with different polarities: certain antioxidants are better soluble in polar solvents such as methanol, water, while cyclohexane or dichloromethane are preferable for isolating lipophilic compounds. Two main approaches may be applied for exhausting isolation of various components from biological material, namely parallel extraction of initial material with different solvents or sequential fractionation with increasing polarity and dielectric constant solvents. The latter approach was applied in our study: non-polar cyclohexane was followed by polar aprotic solvent dichloromethane, the residues were further extracted with polar protic solvent methanol and the process was finalized with boiling water possessing the highest dielectric constant. It is obvious (Table 1) that selected mushrooms are composed of very different classes of substances from the point of view of their solubility in the applied solvents. Thus, the highest total yield of all fractions was obtained from *T. caligatum* (63.15%), while the lowest one from *I. hispidus* (16.48%). Protic solvents possessing high dielectric constant gave remarkably higher extract yields comparing with non-polar and aprotic solvents. It proves that all tested mushroom species contain low amounts of lipophilic constituents. The yields obtained by different solvents are very important characteristics in applying biorefinery concept to biomaterials for their effective, preferably no-waste conversion into the fractions for different applications. For comparison, previously reported yields of methanol (Yang, Lin, & Mau, 2002) and ethanol (Arbaayah & Kalsom, 2013) extracts of *P. ostreatus* were 16.9% and 12.01%, respectively; however, in these studies the initial material was extracted. Sequential extraction of *A. bisporus* with hexane, ethyl acetate and aqueous methanol was also applied previously and the yields were 0.68, 0.65 and 5.84%, respectively (Öztürk et al., 2011). In our study methanol and water yields were remarkably higher. The yield of phenolic and polysaccharide fractions of *X. chrysenteron* from Portugal were reported 12.28 and 27.40% (Heleno et al., 2012); thus, the sum of extracts (39.68%) is similar to the sum of yields (39.93%) obtained in our study for this species.

3.2. Antioxidant potential of different mushroom species

Growing interest in natural antioxidants has led to the development of a large number of assays for evaluating antioxidant capacities of botanical extracts. Since the antioxidant capacity of complex biological extracts is usually determined by a mixture of

various antioxidatively active constituents, which may act by different mechanisms and sometimes possess synergistic effects, the reliability of the evaluation of overall antioxidant potential of any plant material increases by applying several assays (Frankel & Meyer, 2000; Laguerre, Lecomte, & Villeneuve, 2007).

ABTS^{•+} and DPPH[•] scavenging, FRAP, ORAC and TPC assays are the most common methods for determining *in vitro* antioxidant capacity of plant origin substances. Huang, Ou, and Prior (2005) concluded that ORAC, TPC measured with Folin-Ciocalteu reagent and one of the single electron/hydrogen atom transfer assays (SET or HAT) should be recommended for the representative evaluation of antioxidant properties. DPPH[•] scavenging method is mainly attributed to the SET assays; however, quenching of DPPH[•] to form DPPH-H is also possible. Other SET based methods include the TPC assessment using Folin-Ciocalteu reagent, ABTS^{•+} decolourisation assay and ferric ion reducing antioxidant power (FRAP) assay. ORAC assay evaluates radical chain breaking antioxidant activity via HAT and measures antioxidant inhibition induced by peroxy radical oxidation. Following the above mentioned recommendation all these methods were applied for the comprehensive assessment of antioxidant potential of the isolated with different solvents mushroom fractions. To the best of our knowledge such approach is applied for the selected mushroom species for the first time. Moreover, the reports on antioxidant properties of *T. caligatum*, *T. columbetta* and *H. ferrugineum* have not been found in any available literature sources.

To obtain comparable values the results of ABTS^{•+}, DPPH[•], FRAP and ORAC assays were expressed in Trolox (a hydrosoluble analogue of vitamin E) equivalents, i.e. in the amount of Trolox μM possessing similar antioxidant capacity as 1 g edw, while TPC was expressed in mg of gallic acid equivalents per g of edw (Table 2). Remarkable diversity in antioxidant capacity, depending on mushroom species, extraction solvent and assay method, may be clearly observed. Therefore, it is convenient to consistently discuss the effect of these factors in separate sections. For easier assessment of antioxidant potential of different mushroom species, as well as the effects of different solvents and applied antioxidant activity assays (Table 2). The antioxidant characteristics were also summarized by using 'antioxidant scores' of extracts (ASE) and dry mushroom material (ASM) and expressed in the so-called 'comparative integrated units' (ciu), which are explained in 2.5 section.

3.2.1. Antioxidant capacity differences between mushroom species

The highest antioxidant potential demonstrated *I. hispidus* and *P. schweinitzii* extracts, particularly in SET assays, while the extracts isolated from such species as *P. ostreatus* and *A. bisporus* were the weakest antioxidant sources in these assays. For instance, the sum of TEAC of *I. hispidus* extracts in ABTS^{•+} scavenging assay (225.2 $\mu\text{M TE/g}$) was more than 100 times higher comparing to *P. ostreatus* (2.1 $\mu\text{M TE/g}$). These differences were less remarkable in

Table 1
The yields of mushroom extracts isolated by different solvents, % (w/w).

Mushroom species	Cyclohexane	Dichloromethane	Methanol	Water	Total
<i>Phaeolus schweinitzii</i>	3.75 ± 0.30	2.07 ± 0.16	13.91 ± 0.20	4.82 ± 0.20	24.55
<i>Inonotus hispidus</i>	0.40 ± 0.15	0.43 ± 0.25	4.55 ± 0.10	11.10 ± 0.15	16.48
<i>Tricholoma columbetta</i>	1.55 ± 0.05	1.57 ± 0.30	18.74 ± 0.30	25.29 ± 0.25	47.15
<i>Tricholoma caligatum</i>	4.00 ± 0.13	2.79 ± 0.10	36.69 ± 0.25	19.67 ± 0.30	63.15
<i>Xerocomus chrysenteron</i>	2.64 ± 0.25	1.90 ± 0.20	14.16 ± 0.12	21.23 ± 0.17	39.93
<i>Hydrellium ferrugineum</i>	1.42 ± 0.12	1.33 ± 0.12	11.67 ± 0.16	2.94 ± 0.23	17.36
<i>Agaricus bisporus</i>	1.18 ± 0.19	0.89 ± 0.24	16.95 ± 0.25	17.52 ± 0.18	36.54
<i>Pleurotus ostreatus</i>	1.90 ± 0.23	1.38 ± 0.10	10.29 ± 0.10	4.32 ± 0.20	17.89

Results are expressed as a mean ± standard deviation ($n = 3$).

Table 2
Antioxidant activity of extracts isolated from 8 mushroom species by different solvents.

Botanical name	Extract	$\mu\text{M TE/g dw}$				TPC, mg GAE/g dw
		ABTS ^{•+}	DPPH [•]	FRAP	ORAC	
<i>Inonotus hispidus</i>	C	0.10 ± 0.02	0.95 ± 0.13	10.89 ± 0.49	7.50 ± 0.08	4.79 ± 0.36
	D	5.87 ± 1.58	1.72 ± 0.13	24.59 ± 0.64	18.63 ± 3.19	9.70 ± 0.64
	M	54.27 ± 0.46	9.50 ± 0.04	88.31 ± 1.96	290.00 ± 1.00	28.91 ± 1.49
	W	165.00 ± 1.70	8.71 ± 0.04	301.00 ± 2.00	53.06 ± 1.09	41.27 ± 0.86
<i>Tricholoma caligatum</i>	D	4.02 ± 0.40	3.72 ± 0.30	8.75 ± 0.42	69.74 ± 1.98	3.87 ± 0.01
	M	7.44 ± 1.58	0.12 ± 0.02	0.43 ± 0.12	158.00 ± 0.51	4.76 ± 0.60
	W	3.53 ± 1.77	2.04 ± 0.46	17.29 ± 0.38	5.83 ± 1.03	5.89 ± 0.24
<i>Tricholoma columbetta</i>	C	3.50 ± 0.12	0.44 ± 0.11	12.02 ± 0.35	155.00 ± 1.00	6.25 ± 0.20
	D	3.31 ± 0.18	0.67 ± 0.64	6.52 ± 0.62	135.00 ± 3.00	4.74 ± 0.06
	M	0.48 ± 0.27	1.24 ± 0.48	0.91 ± 0.61	64.63 ± 3.57	6.60 ± 3.50
	W	1.60 ± 0.13	0.59 ± 0.26	10.72 ± 0.17	10.83 ± 0.65	5.18 ± 0.28
<i>Phaeolus schweinitzii</i>	C	4.82 ± 0.70	3.65 ± 0.92	0.45 ± 0.07	33.55 ± 1.89	7.07 ± 0.49
	D	4.17 ± 0.21	1.33 ± 0.04	10.95 ± 0.25	42.74 ± 2.55	9.38 ± 0.41
	M	164.00 ± 1.00	9.62 ± 0.03	109.00 ± 3.00	340.00 ± 3.00	31.88 ± 1.67
	W	7.93 ± 1.35	8.89 ± 2.84	97.19 ± 0.85	45.63 ± 2.70	8.20 ± 0.33
<i>Xerocomus chrysenteron</i>	C	0.89 ± 0.46	0.03 ± 0.01	8.12 ± 0.14	26.47 ± 1.44	6.44 ± 0.31
	D	3.54 ± 0.28	2.44 ± 0.04	7.52 ± 0.49	16.35 ± 0.04	5.74 ± 0.14
	M	1.37 ± 0.08	0.51 ± 0.16	3.64 ± 0.35	49.33 ± 3.29	4.96 ± 0.18
	W	1.45 ± 0.36	0.69 ± 0.07	9.92 ± 1.84	11.85 ± 0.43	6.56 ± 0.12
<i>Hydnellum ferrugineum</i>	C	1.77 ± 1.47	0.05 ± 0.02	16.95 ± 0.49	38.41 ± 1.97	7.22 ± 0.53
	D	1.08 ± 0.53	3.02 ± 0.34	18.02 ± 0.42	90.65 ± 1.16	6.92 ± 0.41
	M	1.38 ± 0.21	0.60 ± 0.06	11.71 ± 0.99	74.02 ± 3.06	8.05 ± 0.23
	W	10.71 ± 0.69	1.65 ± 0.15	16.99 ± 0.07	63.54 ± 3.34	13.31 ± 0.07
<i>Agaricus bisporus</i>	C	2.06 ± 0.24	1.53 ± 0.16	10.65 ± 0.64	17.33 ± 0.58	4.21 ± 0.05
	D	0.94 ± 0.16	0.32 ± 0.11	11.62 ± 0.17	34.55 ± 3.16	4.27 ± 0.06
	M	1.41 ± 0.38	0.13 ± 0.03	2.09 ± 0.12	65.84 ± 3.00	4.23 ± 0.12
	W	0.82 ± 1.82	0.15 ± 0.04	1.35 ± 1.34	30.92 ± 1.61	4.64 ± 0.03
<i>Pleurotus ostreatus</i>	C	0.10 ± 0.04	0.08 ± 0.07	15.49 ± 2.62	115 ± 4.00	4.73 ± 0.10
	D	0.55 ± 0.19	1.52 ± 0.37	8.42 ± 1.34	90.73 ± 2.32	5.67 ± 1.30
	M	0.65 ± 0.04	0.74 ± 0.21	1.49 ± 0.83	76.11 ± 1.59	5.32 ± 0.98
	W	0.80 ± 0.18	0.14 ± 0.05	6.02 ± 2.05	1.73 ± 0.54	4.26 ± 0.01

C, cyclohexane; D, dichloromethane; M, methanol; W, water; results are expressed as a mean ± standard deviation ($n = 4$); cyclohexane extracts of *T. caligatum* was not assayed.

other SET assays, while in ORAC assay the values varied from 104.0 (*X. chrysenteron*) to 461.9 $\mu\text{M TE/g}$ (*P. schweinitzii*). TPC was from 14.52 (*T. caligatum*) to 84.57 mg GAE/g (*I. hispidus*). However, extract yields were dependent both on mushroom species and extraction solvent, therefore TPC values obtained for extracts were recalculated for 1 g of mushroom dry weight (mdw), taking into account how much of TPC is extracted with each solvent. The TPC values expressed in this way are presented in Fig. 1: they were from 0.90 mg GAE/g mdw (*P. ostreatus*) to 5.96 mg GAE/g mdw

(*I. hispidus*). In general the TPC values in most cases were in agreement with antioxidant capacity values obtained in other assays.

Taking into account all measured characteristics, the ASMs of wild mushrooms expressed in ciu/g mdw may be located in the following decreasing order (Fig. 2): *P. schweinitzii* (97.06) > *I. hispidus* (78.99) > *T. caligatum* (69.95) >> *T. columbetta* (23.54) > *H. ferrugineum* (15.28) > *X. chrysenteron* (14.35). Commercial species *A. bisporus* and *P. ostreatus* were of inferior

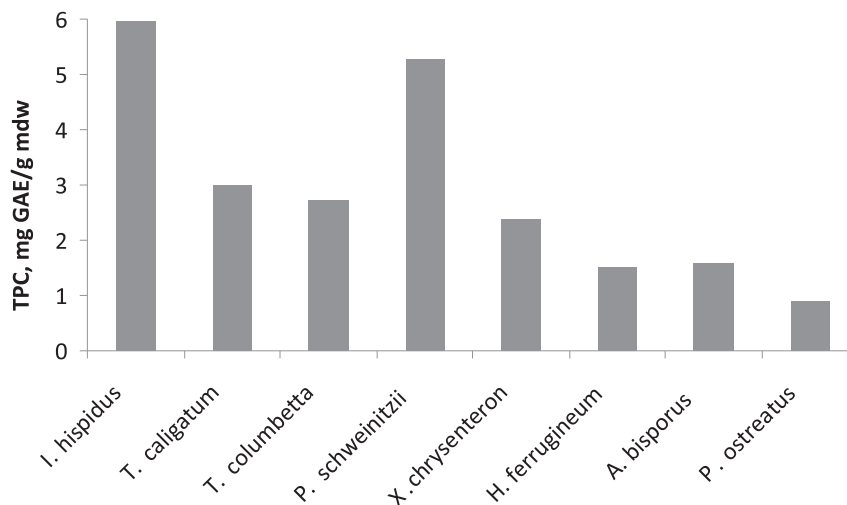


Fig. 1. Total content of phenolic compounds (TPC) in mushrooms.

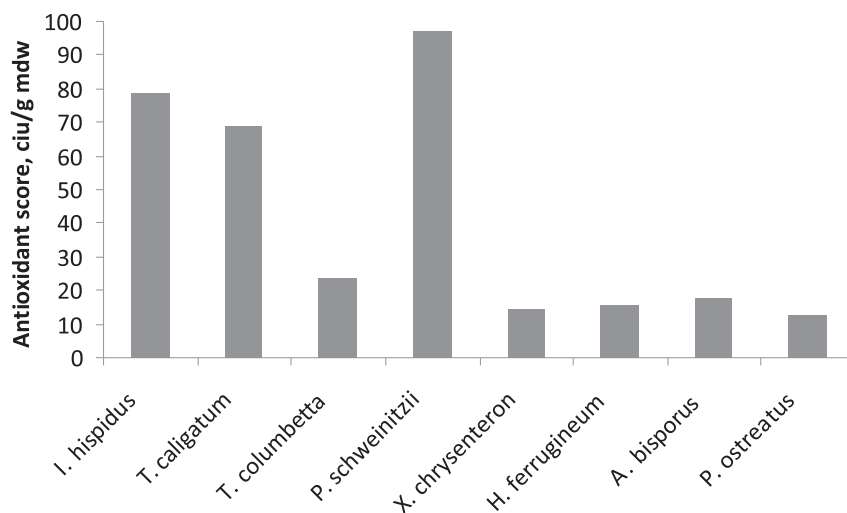


Fig. 2. Antioxidant scores of mushroom species (ASM) integrating antioxidant activity values in ABTS^{•+}, DPPH[•], FRAP and ORAC assays and extract yields.

antioxidant potential comparing with the majority of studied wild mushrooms species; their ASMs were 18.02 and 12.37 ciu/g mdw, respectively. It was previously reported that methanol extract isolated from *P. ostreatus* was stronger DPPH[•] and OH[•] scavenger and possessed better reducing properties comparing with other 5 tested commercial mushroom species (Yang et al., 2002); however, in the mentioned study antioxidant indicators were expressed in percentage of scavenged radicals and therefore are difficult to compare with our results. More effective mushrooms species were reported to contain higher amounts of secondary metabolites such as phenolics exerting multiple biological effects including antioxidant activity (Kim et al., 2008).

3.2.2. Effect of extraction solvent

Generally polar solvents are most frequently used for the extraction of antioxidants from botanicals containing polyphenolics as the main antioxidatively active compounds; however, some plant origin materials may also contain lipophilic compounds such as tocopherols, carotenoids, terpenoids and the use of different polarity solvents may provide more comprehensive information on their antioxidant potential, particularly in case of less studied mushroom species. Our results clearly demonstrate (Table 1) that distribution of antioxidatively active constituents in the fractions isolated with different solvents is highly dependent on mushroom species. The extracts isolated from *I. hispidus* and *P. schweinitzii* (the species possessing the highest antioxidant potential) with protic solvents methanol and water were remarkably stronger antioxidants than cyclohexane and dichloromethane extracts of the same species in all assays, while for other species the results are more complicated. For instance, cyclohexane extract of the well-known commercial *A. bisporus* mushroom was stronger antioxidant in all assays except for ORAC, while the TPC values were quite similar for all extracts. However, it should be mentioned that this species was characterized as possessing weak antioxidant potential. Dichloromethane extract of *X. chrysenteron* was stronger antioxidant in SET assays, except for FRAP and TPC values; however, in ORAC assay methanol fraction was 3 times stronger than dichloromethane extract.

It is interesting noting that *P. ostreatus* cyclohexane extract was strongest antioxidant in FRAP and ORAC assays, while its reducing power reflected by the TPC values was quite equally distributed in all fractions isolated from this species. Previously reported TPC value of methanol extract isolated from *P. ostreatus* was

15.7 ± 0.1 mg/g (Yang et al., 2002), i.e. 3 times higher than in our study measured methanol extract; however, the extract in previous study was obtained from the whole material, while in our study methanol was used for reextracting the residue after cyclohexane and dichloromethane extraction. In another study (Yim, Chye, Tan, Ng, & Ho, 2010) approx. 8 mg of phenolics (in tannic acid equivalents) in 1 g dw water extract were determined. The sum of TPC in all organic extracts obtained in our study was 19.98 mg GAE/g, i.e. similar as in the previously assayed methanol extract (Yang et al., 2002).

Water is a preferable solvent in terms of toxicity and availability; however, it is not always sufficiently efficient for the isolation for plant bioactive compounds. Our study shows that water may be useful solvent for the extraction of remaining antioxidants from some mushroom species after applying different polarity organic solvents. For instance, water extract of *I. hispidus* was strongest antioxidant in ABTS^{•+}, FRAP and TPC assays; however, it was almost 6 times weaker in ORAC assay compared to methanol fraction. It is also important noting that the yield of water extract from *I. hispidus* was remarkably higher than the yields obtained with other solvents; water fraction constituted 67% of the total extractives. Water extracts of other species were also remarkably less effective in ORAC assay, except for *H. ferrugineum*, when the difference between methanol and water fractions constituted only 14%. It should be noted that water extracts were obtained by boiling the residues of extractions with organic solvents and in this case some hydrolysis and other processes involving chemical changes may occur in extraction material. Cyclohexane and dichloromethane fractions were several times weaker radical scavengers than polar methanol and water extracts. It is in agreement with many previously published results showing that polar solvents extract more antioxidants from botanicals than lower polarity solvents (Brahmi, Mechri, Dabbou, Dhibi, & Hammami, 2012). ASEs were calculated for the extracts isolated with different solvents for comparative assessment of the effectiveness of each solvent for the tested mushroom species. They may be located in the following decreasing order (ASE in ciu/g edw is indicated in the brackets):

I. hispidus: W (569) > M (471) > D (61) > C (24);
P. schweinitzii: M (654) > W (168) > D (69) > C (50);
T. columbetta: C (177) > D (150) > M (74) > W (29);
H. ferrugineum: D (120) > W (106) > M (96) > C (64);
P. ostreatus: C (135) > D (107) > M (84) > W (13);

T. caligatum: M (171) > D (90) > W (35);
A. bisporus: M (74) > D (52) > W (38) > C (36);
X. chrysenteron: M (60) > C (42) > D (36) > W (30).

However, it should be noted that in this case the scores were calculated by summing the values measured for 1 g of extracts dw and are not similar to ASM which were calculated in $\mu\text{g}/\text{g}$ mdw and presented in Fig. 2; the latter reflect the antioxidant potential of all extracted fractions plus their yields in all assays.

3.2.3. Effects of assay method

Antioxidant activity values obtained by using different evaluation assays are in a very wide range, they depend both on mushroom species and extracted fraction. Generally the highest values were obtained in ORAC assay; their sum from all fractions were from 104 (*X. chrysenteron*) to 462 $\mu\text{M TE/g}$ (*P. schweinitzii*). FRAP values were from 25 (*P. ostreatus*) to 425 $\mu\text{M TE/g}$ (*I. hispidus*); TEAC values in ABTS⁺ scavenging assay were from 2.1 (*P. ostreatus*) to 225 $\mu\text{M TE/g}$ (*I. hispidus*), while the lowest values were measured in DPPH[•] scavenging assay, from 2.1 (*A. bisporus*) to 23.5 $\mu\text{M TE/g}$ (*P. schweinitzii*). Strong correlation was observed between total phenolics and DPPH[•] ($R^2 = 0.8969$) and ABTS⁺ scavenging capacity ($R^2 = 0.9255$), confirming that phenolic compounds are important contributors to the antioxidant properties of these extracts. However, the correlations between TPC and ORAC ($R^2 = 0.7712$), as well as between TPC and FRAP ($R^2 = 0.7573$) were weaker.

Several reasons may be considered to explain the obtained differences between the applied assays. Although the principle of the applied radical scavenging or reduction assays are based on SET and/or HAT, the peculiarities of reaction mechanisms in each assay are different; they may largely depend on reaction media, pH, the structure of antioxidative compounds present in the extracts, their interactions and other factors. For instance, Zan et al. (2011), reported that in ABTS⁺ scavenging assay, 5 from *I. hispidus* methanol extract isolated compounds exhibited significant activity, from 12.71 ± 3.57 to 59 ± 9.70 $\mu\text{M TE}/\mu\text{M}$ compound. These findings support our results indicating high ABTS⁺ scavenging capacity of *I. hispidus* water and methanol fractions. Thus, the TEAC values in ABTS⁺ assay of the all studied mushrooms measured in the all extracts were of the following decreasing order: *I. hispidus* > *P. schweinitzii* > *T. caligatum* > *H. ferrugineum* > *T. columbetta* > *X. chrysenteron* > *A. bisporus* > *P. ostreatus*. However, the TEAC values recalculated for 1 g mdw, which also consider extract yields would be in different order (TEAC in $\mu\text{g}/\text{g}$ mdw in brackets): *P. schweinitzii* (23.45) > *I. hispidus* (20.81) >> *T. caligatum* (3.54) > *T. columbetta* > (0.60) > *X. chrysenteron* (0.59) > *H. ferrugineum* (0.52) > *A. bisporus* (0.41) > *P. ostreatus* (0.11). Antioxidant properties of *A. bisporus* extracts sequentially isolated by different polarity solvents were evaluated previously by measuring their effective concentrations EC₅₀; aqueous methanol fraction was stronger antioxidant comparing to hexane and ethyl acetate extracts in β -carotene linoleic acid co-oxidation system, DPPH[•], ABTS⁺ and CUPRAC assays (Öztürk et al., 2011). Although we used different extraction procedure and antioxidant activity assays, some agreement in the obtained results may be observed, particularly in case of ABTS⁺ scavenging assay, when in both studies methanol and water extracts were remarkably stronger antioxidants and ABTS⁺ values were higher than DPPH[•] values. RSC of water fraction of *P. ostreatus* was also measured by Yim et al. (2010); however, it was expressed in percentage of scavenged radicals, i.e. the units which are not applicable for comparison purposes.

The sum of values measured in DPPH[•] scavenging assay of all extracts were of the following decreasing order: *P. schweinitzii* > *I. hispidus* > *T. caligatum* > *H. ferrugineum* > *X. chrysenteron* > *T. columbetta* > *P. ostreatus* > *A. bisporus*. Again integrated DPPH[•]

scavenging values recalculated for 1 g mdw would be in slightly different order ($\mu\text{g}/\text{g}$ mdw in brackets): *P. schweinitzii* (1.93) > *I. hispidus* (1.41) > *T. caligatum* (0.55) > *T. columbetta* (0.40) > *X. chrysenteron* (0.27) > *H. ferrugineum* (0.16) > *P. ostreatus* (0.10) > *A. bisporus* (0.07). Previous reported values for *A. bisporus*, IC₅₀ = 0.38 mg/mL for ethanol extract (Liu, Jia, Kan, & Jin, 2013) and IC₅₀ = 0.988 ± 0.3 mg/mL for methanol extract (Öztürk et al., 2011) are difficult to compare with our results obtained for *A. bisporus* methanolic and water fractions (0.13–0.14 $\mu\text{M TE/g}$). Our study shows that DPPH[•] scavengers from this species are more effectively extracted with cyclohexane (1.53 $\mu\text{M TE/g}$), whereas in case of other studied species cyclohexane fractions were weaker DPPH[•] scavengers, except for dichloromethane fraction isolated from *P. schweinitzii*, which was almost 3 times weaker in this assay than cyclohexane fraction. DPPH[•] scavenging capacity was also recently reported for *P. ostreatus* and it was shown that it is dose-dependent (Mishra et al., 2013). Effective DPPH[•] scavenging concentration EC₅₀ of *X. chrysenteron* methanol/water extract was 2.06 ± 0.46 mg/mL (Heleno et al., 2012), while methanol fraction, depending on extract concentration, inhibited from 27.42 ± 1.23 to $89.61 \pm 0.10\%$ DPPH[•] (Sarikurku et al., 2008).

The third method used to evaluate antioxidant potential of mushroom species was ferric ion reducing ability (FRAP). In case of this assay extract's efficiency was in the following decreasing order: *I. hispidus* (37.58) > *P. schweinitzii* (20.08) > *H. ferrugineum* (2.35) > *P. ostreatus* (0.82) > *T. columbetta* (3.17) > *X. chrysenteron* (2.98) > *T. caligatum* (3.80) > *A. bisporus* (0.73). However, the integrated FRAP values calculated for 1 g mdw as it is indicated in the brackets were in different order. FRAP was also used in some other studies of mushrooms; however, their data is difficult to compare due to different units used to express the data. Metal chelating ability of *P. ostreatus* (Mishra et al., 2013) and *X. chrysenteron* (Sarikurku et al., 2008) methanol extracts was reported previously as well, however, it was also expressed in relative units. *A. bisporus* demonstrated strong Fe²⁺ ion chelating capacity: EC₅₀ = 310.00 ± 0.87 $\mu\text{g}/\text{mL}$ (Öztürk et al., 2011). Chelating agents may act as secondary antioxidants by reducing redox potential and stabilizing the oxidised forms of metal ions (Mishra et al., 2013).

Finally, the antioxidant activity of mushrooms was evaluated using ORAC assay; the values for investigated species are located in the following decreasing order considering the sum of values in all extracts (ORAC values calculated for 1 g mdw are presented in the brackets): *P. schweinitzii* (51.60) > *I. hispidus* (19.19) > *T. columbetta* (19.37) > *P. ostreatus* (11.34) > *H. ferrugineum* (12.26) > *T. caligatum* (61.06) > *A. bisporus* (16.81) > *X. chrysenteron* (10.51). Methanol fractions were strongest antioxidants in ORAC assay, except for *T. columbetta* (cyclohexane and dichloromethane fractions were superior), *H. ferrugineum* and *P. ostreatus*, when dichloromethane extracts were stronger peroxy radical inhibitors. The lipophilic and hydrophilic ORAC values of *P. ostreatus* and *A. bisporus* (white button) acetone/water extracts were reported 5.67 and 49.67 (total 55.34); 6.33 and 80.00 (total 86.33) $\mu\text{M TE/g}$ dw of mushroom, respectively (Dubost, Ou, & Beelman, 2007). By recalculating our results from 1 g of edw to 1 g of mdw we obtained 11.34 $\mu\text{M TE/g}$ for *P. ostreatus* and 16.81 $\mu\text{M TE/g}$ for *A. bisporus*, i.e. remarkably lower, comparing with the results reported in the above cited reference. ORAC value of ethanol fraction of *A. bisporus* reported in the same article was 86.33 $\mu\text{M TE/g}$ dw, i.e. higher than the ORAC of methanol (65.84 ± 1.59 $\mu\text{M TE/g}$ dw) and water (30.92 ± 1.61 $\mu\text{M TE/g}$ dw) fractions determined in our study; however, the sum of ORAC values in all extracts measured in our study was 148.64 $\mu\text{M TE/g}$ dw indicating that powerful scavengers of ROO[•] radicals are also present in lower polarity fractions.

The values measured with Folin–Ciocalteu reagent and expressed in gallic acid or other phenolic compound are generally

accepted as representing the total phenolic content (TPC) although it is not fully correct: Folin–Ciocalteu reagent reacts not only with phenolics but with other reducing ability possessing compounds in the reaction system (Huang et al., 2005). Consequently, the term TPC may be used rather conditionally; however, for the convenience we are using this term in our study. Thus, the integrated TPC values (their sum in the all extracts) were distributed in the following decreasing order: *I. hispidus* > *P. schweinitzii* > *H. ferrugineum* > *X. chrysenteron* > *T. columbetta* > *P. ostreatus* > *A. bisporus* > *T. caligatum*. It is interesting noting that only methanol and water fractions of *I. hispidus*, methanol fraction of *P. schweinitzii* and water fraction of *H. ferrugineum*, contained remarkably higher concentrations of TPC than the fractions isolated from the same species with other solvents. For instance, the TPC values in different extracts of commercial mushrooms *A. bisporus* and *P. ostreatus* were in the range of 4.21–4.64 and 4.26–5.67 mg GAE/g, respectively. TPC in acetone/water fraction of *A. bisporus* and *P. ostreatus* was previously reported 8.00 ± 0.48 and 4.27 ± 0.69 mg GAE/g mdw (Dubost et al., 2007); we obtained lower values of TPC from these species (Fig. 1). Previously reported TPC in *X. chrysenteron* (36.28 ± 0.5 mg GAE/g extract) methanol/water fraction (Heleno et al., 2012) is higher than the sum of TPC in all extracts obtained from this species in our study. In general, we obtained the values of the same order comparing to the previously published data, while the differences are reasonable, so far as the content of polyphenolics in the same species may vary depending on cultivar, harvesting time, climatic conditions and other factors.

3.3. Antibacterial properties of plant extracts

Evaluation of antioxidant activity of extracts isolated from the selected mushroom species by different solvents revealed remarkable variability in the obtained values. It is known that many antioxidatively active compounds may also possess different effects against microorganisms. Therefore, the study was continued for the preliminary screening of antimicrobial properties of the extracts against two bacteria and 1 yeast species. The results obtained for 32

extracts isolated from 8 mushroom species are summarized in Table 3 listing the inhibition zones in the agar diffusion assay at different applied concentrations. In general, Gram-negative *P. aeruginosa* were more sensitive to the applied extracts than Gram-positive *B. cereus*. For instance, the latter bacterium was not inhibited by any of the extracts concentrations of 0.1 and 1 mg/mL. Comparing the solvents, methanol extracts in most cases were the strongest antimicrobial agents, whereas water fractions possessed the weakest inhibitory activity in case of all mushrooms (cyclohexane and dichloromethane extracts of *P. ostreatus* also did not inhibit tested microorganisms at any applied concentration).

Comparing mushrooms species, it may be observed that the fractions of *I. hispidus* isolated with different solvents possessed antibacterial activity against 2 bacteria and 1 yeast species in the agar diffusion assay, however water extract was effective only against *P. aeruginosa*. All extracts of *P. schweinitzii* isolated with cyclohexane, dichloromethane and methanol inhibited tested microorganism. For instance, the largest inhibition zones were observed for methanol fraction of *P. schweinitzii* against *P. aeruginosa* (17 ± 0.5 mm), *B. cereus* (16 ± 1.9 mm), and *C. albicans* (15 ± 0.0 mm). Strong effect demonstrated methanol extract of *I. hispidus* against *P. aeruginosa* (17 ± 1.5 mm) and *C. albicans* (15 ± 0.5 mm). It is interesting noting that these two above mentioned species also possessed the highest antioxidant potential, comparing with other studied species, which were remarkably less active in antimicrobial tests. *T. columbetta* methanol fraction showed strong effect against *C. albicans* (17 ± 2.0 mm), *T. caligatum*, *X. chrysenteron*, *A. bisporus*, *P. ostreatus* methanol fractions showed strong effect against *C. albicans* (15–14 mm); *H. ferrugineum*, *A. bisporus* also showed strong effect against *P. aeruginosa* (15 mm).

MIC values which are evaluated by diluting the extracts and measuring the lowest inhibitory concentrations are important indicators of antimicrobial activity (Table 3). None of the tested extracts was active at the concentration of 0.01 mg/mL. The lowest MIC values (0.1 mg/mL) against *C. albicans* were determined for cyclohexane, dichloromethane and methanol fractions of *I. hispidus* and *P. schweinitzii*; cyclohexane and methanol fractions of

Table 3
Antimicrobial activity of mushroom extracts, in mm of inhibition zones.

Botanical name	Fraction	<i>Candida albicans</i>				<i>Bacillus cereus</i>		<i>Pseudomonas aeruginosa</i>		
		20 mg/mL	10 mg/L	1 mg/mL	0.1 mg/mL	20 mg/mL	10 mg/mL	20 mg/mL	10 mg/mL	1 mg/mL
<i>I. hispidus</i>	C	10 ± 1.1	11 ± 2.0	8.5 ± 0.7	8.0 ± 0.7	8.0 ± 0.5	7.0 ± 1.0	7.5 ± 0.5	7 ± 0.0	6.5 ± 0.5
	D	9.5 ± 0.7	9.0 ± 0.5	8.5 ± 0.7	7.0 ± 0.4	7.5 ± 0.5	6.5 ± 1.0	9.5 ± 0.5	8.5 ± 1.0	7.2 ± 1.2
	M	15 ± 0.5	13 ± 0.0	7.0 ± 0.9	6.0 ± 0.0	13 ± 0.1	10 ± 2.1	17 ± 1.5	13 ± 1.0	6.5 ± 0.5
	W	n.a	n.a	n.a	n.a	n.a	n.a	12 ± 1.3	9 ± 0.3	n.a
<i>T. caligatum</i>	C	10 ± 0.0	6.5 ± 0.0	n.a	n.a	11 ± 1.5	n.a	8.5 ± 0.5	6.5 ± 0.5	n.a
	D	6.0 ± 0.0	n.a	n.a	n.a	6.0 ± 0.0	n.a	9.0 ± 0.0	n.a	n.a
	M	15 ± 1.0	13 ± 0.0	7.0 ± 0.0	n.a	11 ± 2.1	10 ± 1.0	13 ± 0.0	9.5 ± 1.0	n.a
<i>T. columbetta</i>	C	n.a	n.a	n.a	n.a	8.0 ± 2.5	n.a	9.0 ± 1.0	7.0 ± 0.0	n.a
	D	n.a	n.a	n.a	n.a	11 ± 0.0	n.a	9.0 ± 1.0	9.0 ± 1.5	n.a
	M	17 ± 2.0	13 ± 1.5	n.a	n.a	13 ± 1.0	12 ± 0.0	14 ± 1.0	10 ± 1.5	n.a
<i>P. schweinitzii</i>	C	10 ± 1.0	8.5 ± 0.5	6.0 ± 1.0	6.0 ± 1.5	11 ± 0.0	9.0 ± 2.2	7 ± 1.2	6.0 ± 0.5	n.a
	D	8.0 ± 1.5	7.0 ± 0.0	7.0 ± 1.0	6.5 ± 1.5	16 ± 2.3	10 ± 0.9	9 ± 0.5	n.a	n.a
	M	15 ± 0.0	14 ± 0.5	9.5 ± 0.5	7.0 ± 0.0	16 ± 1.9	15 ± 1.5	17 ± 0.5	16 ± 1.0	13 ± 1.4
<i>X. chrysenteron</i>	C	n.a	n.a	n.a	n.a	n.a	n.a	7.0 ± 0.5	6.0 ± 1.0	6.0 ± 1.0
	D	9.0 ± 0.5	7.0 ± 1.0	7.0 ± 5.0	6.5 ± 2.5	n.a	n.a	9.5 ± 0.9	9.0 ± 0.5	8.0 ± 1.5
	M	15 ± 0.9	14 ± 1.1	n.a	n.a	13 ± 0.5	n.a	10 ± 1.0	9.0 ± 0.5	n.a
<i>H. ferrugineum</i>	C	9.0 ± 1.0	8.0 ± 1.3	7.0 ± 0.5	7.0 ± 0.0	n.a	n.a	n.a	n.a	n.a
	D	n.a	n.a	n.a	n.a	6.5 ± 1.3	6.0 ± 1.0	n.a	n.a	n.a
	M	14 ± 1.0	143 ± 1.1	9.0 ± 0.5	7.0 ± 1.0	n.a	n.a	15 ± 1.3	14 ± 1.1	n.a
<i>A. bisporus</i>	C	n.a	n.a	n.a	n.a	14 ± 0.0	10 ± 2.3	n.a	n.a	n.a
	D	n.a	n.a	n.a	n.a	13 ± 0.5	12 ± 0.4	15 ± 0.0	14 ± 0.7	n.a
	M	15 ± 1.2	13 ± 0.0	n.a	n.a	n.a	n.a	12 ± 2.1	10 ± 1.5	6.5 ± 1.4
<i>P. ostreatus</i>	M	15 ± 1.4	13 ± 1.0	6 ± 1.3	n.a	10 ± 1.2	9.0 ± 1.0	10 ± 1.1	8.0 ± 1.4	n.a

C, cyclohexane; D, dichloromethane; M, methanol; W, water; n.a., not active; results are expressed as a mean ± standard deviation ($n = 3$); none of the extracts was effective against *Bacillus cereus* at 0.1 and 1 mg/mL; only dichloromethane extract of *X. chrysenteron* formed inhibition zone (6.0 ± 1.5 mm) against *P. aeruginosa* at 0.1 mg/mL; the fractions which did not form any inhibition zone at all applied concentrations are excluded from the table.

H. ferrugineum, and dichloromethane fraction of *X. chrysenteron*. *B. cereus* was more resistant and MIC for different fractions was not lower than 10 mg/mL. Dichloromethane fraction of *X. chrysenteron* was most efficient against *P. aeruginosa* (MIC = 0.1 mg/mL), 7 fractions from various mushrooms had MIC of 1 mg/mL, while the majority of other fractions demonstrated MIC of 10 or 20 mg/mL. Only few reports on antimicrobial activity of some species tested in our studies are available. Previously reported MICs for *P. ostreatus* ethanol extract were 1.25 mg/mL against *C. albicans*, 2.5–20 mg/mL against *P. aeruginosa*, 2.5–12.5 mg/mL against *B. cereus*; antimicrobial activity was shown to be dependent on nitrogen source (Vamanu, 2012). In reported studies methanol extract isolated from *A. bisporus* showed similar antimicrobial activity against *C. albicans* 16 ± 0 SD and *B. cereus* 21 ± 0 (Öztürk et al., 2011).

4. Conclusions

Antioxidant potential of studied mushrooms was found to be in a rather wide range: the differences were observed between the tested species as well as between the fractions isolated by different solvents. Assay method was also important factor in determining antioxidant properties of mushroom extracts, therefore ORAC, TPC measured with Folin-Ciocalteu reagent and one of the single electron transfer assays, as recommended by Huang et al. (2005), should be performed for the representative evaluation of antioxidant properties.

The fractions isolated with methanol and water from *P. schweinitzii* and *I. hispidus* were most powerful antioxidants almost in all tested assays. Antimicrobial activity of mushroom extracts expressed in minimal inhibitory concentration was also found to be in a wide range. The results suggest that different classes of antioxidatively active constituents may be present in the studied mushroom species. Remarkable differences in antioxidant and antimicrobial properties of extracts isolated with different polarity solvents also indicate about the presence of high variety 'mycochemicals' in various mushroom species. Considering that comparatively low number of compounds have been identified in the selected mushroom species until now, the results may foster further studies of mushroom species aimed at searching of new bioactive compounds, which might be of interest for various applications, such as ingredients of functional foods, nutraceuticals, pharmaceuticals, and cosmetics.

Acknowledgements

The authors are deeply grateful for mycologist Mr. Louis Chavart and "Association Mycologique de Toulouse" for mushrooms samples. Also the authors are thankful to Dr. Zanda Kruma in Latvia University of Agriculture for the antibacterial tests supervision. This study was supported by Research Council of Lithuania (SVE-06/11) and by Midi-Pyrénées Regional Council and SMI (International mobility support) program of INP Toulouse. It was performed in the framework of COST Action TD1203.

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Comprehensive Evaluation of Antioxidant Potential of Coastal Dune Mushroom Species from the South-West of France

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ABSTRACT: Numerous mushroom species are used for food and medicinal purposes, however many species, which may contain bioactive compounds, remain underinvestigated. In this study, the antioxidant properties of extracts sequentially isolated with cyclohexane, dichloromethane and methanol from 25 coastal dune mushroom species collected in the south-western region of France were evaluated by radical scavenging capacity, ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC) and Folin–Ciocalteu total phenolic content (TPC) methods. Overall, the antioxidant potential of dried mushrooms were assessed by using integrated antioxidant scores. The highest antioxidant capacity values demonstrated the *Cortinarius infractus*, *Agaricus coniferarum*, *Agaricus menieri* and *Agaricus freirei* species. The results obtained may foster further studies of the selected mushroom species for their nutritional and medicinal valorization.

KEYWORDS: dune mushrooms, coastal mushrooms, extracts, antioxidant activity

ABBREVIATIONS: **AAPH:** 2,2'-azobis(2-amidino-propane) dihydrochloride; **ABTS:** 2,20-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; **ASE:** 'antioxidant score' of extract; **ASM:** antioxidant score of mushroom; **ciu:** comparative integrated unit; **DPPH:** 2,2-di-phenyl-1-picrylhydrazyl hydrate radical; **edw:** extract dry weight; **FL:** fluorescein; **FRAP:** ferric reducing antioxidant power; **GAE:** gallic acid equivalents; **mdw:** mushroom dry weight; **OAP:** overall antioxidant power; **ORAC:** oxygen radical absorbance capacity; **RSC:** radical scavenging capacity; **PBS:** phosphate buffered saline; **TE:** Trolox equivalents; **TEAC:** Trolox equivalent antioxidant capacity; **TPC:** total phenolic content; **TPTZ:** 2,4,6-tripyridyl-s-triazine.

I. INTRODUCTION

Natural dietary antioxidants are believed to assist in maintaining good health and preventing various disorders.¹ Antioxidants are present in all biological systems, including mushrooms. Many mushroom species have been a part of the human diet for centuries, due to their nutritional and flavor properties and health benefits. Although, modern science based knowledge about mushroom's bioactivities and chemical composition has been obtained quite recently, a number of mushroom species have been reported during the last decade to possess multifunctional properties,² including antioxidant activities.³⁻⁵ Mushrooms biosynthesize various bioactive molecules, such as phenolic compounds, polyketides, terpenes and steroids.⁶ Some naturally occurring constituents identified in mushrooms served as models for clinically

proven drugs.⁷ However, the assessment of possibilities of mushroom applications in the nutraceutical and functional food area is quite recent. From this point of view, the mushrooms exhibiting health benefits and at the same time possessing delicate flavor properties might be good ingredients in developing medicinal foods.

For increasing practical exploration of mushroom diversity in various regions, mushrooms should be more comprehensively characterised by various activity guided assays. This study is focused on coastal-dune mushrooms growing wild in south-western France, as previously performed studies on the selected species are rather scarce. The following articles were published on the composition and bioactivities of some mushroom species: volatile chemical composition of *Agaricus pseudopratensis*;⁸ microbial and antioxidant activity of *Agaricus devoniensis*;⁹ antitumor activity of *Agaricus fissuratus*;¹⁰ chemical, antioxidant and nutritional value of *Boletus impolitus*;¹¹ antimicrobial potential¹² and monoterpene volatile compounds¹³ of *Boletus lupinus*; carotenoids of *Boletus luridus*;¹⁴ active anti-osteoporosis ergosterol derivative¹⁵ and antimicrobial activity¹⁶ of *Tricholoma auratum*; aromatic derivatives of *Tricholoma caligatum*;¹⁷ volatile compounds¹⁸, chemical composition, antioxidant and antimicrobial properties of *Clitocybe odora*;^{19,20} indole alkaloids²¹ and alkaloid activity against Alzheimer's disease²² of *Cortinarius infractus*.

Regardless of the above-cited articles, the reports on the antioxidant activity of the selected mushrooms in this study are rather scarce; previously published data is particularly lacking a more comprehensive approach. Therefore, the aim of the present work was to evaluate the antioxidant potential of the selected 25 mushroom species by applying a more systematic approach. It was achieved by a comprehensive evaluation of mushroom extracts sequentially isolated with cyclohexane, dichloromethane, methanol and water by using RSC, ORAC, FRAP and TPC assays.

II. MATERIALS AND METHODS

A. Mushroom samples

Wild mushroom fruiting bodies were collected in the South-West of France during the autumn of year 2011 (Table 1). Taxonomic identification was performed by the Research Unit "Mycology and Food Safety" at the INRA Research Centre of Bordeaux-Aquitaine. Freeze-dried mushrooms were ground through a 1.5-mm sieve and stored in air-tight plastic bags.

B. Materials

Gallic acid, DPPH[•] (95%), anhydrous Na₂CO₃, Trolox (97%), ABTS, FL and AAPH were from Sigma-Aldrich (Steinheim, Germany); 2.0M Folin-Ciocalteu phenol reagent, KCl, NaCl, Na₂HPO₄, Na₂CO₃ and K₂S₂O₈ from Merck (Darmstadt, Germany); KH₂PO₄ from Jansen Chimica (Beerse, Belgium); methanol, 98% acetic acid from Lachema (Brno, Czech Republic), ethanol (96.6%) from Stumbras (Kaunas, Lithuania); 2,4,6-tripyridyl-s-triazine (TPTZ) from Fluka Chemicals (Steinheim, Switzerland).

C. Extraction Procedure

HPLC grade cyclohexane, dichloromethane and methanol were used to fractionate soluble compounds in ascending polarity, by sequentially extracting 2-100g (depending on material availability) ground mushrooms in a Soxhlet extractor for 5 h. The solvents after extraction were removed in a vacuum rotary evaporator.

D. Antioxidant Activity Assays

Antioxidant activity was evaluated using previously reported methods.²³ Briefly, ABTS^{•+} and DPPH[•] scavenging, ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) were determined from the calibration curve built with 50–1000 $\mu\text{M/L}$ solutions of Trolox and expressed in μM Trolox equivalents per g of extract dry weight ($\mu\text{M TE/g edw}$) and calculated as follows:

$$TEAC \left(\frac{\mu\text{M}}{\text{g}} \right) = \frac{TE_s}{1000} \times \frac{V_s}{m_s}$$
$$TE_s$$
 – antioxidant activity of sample, V_s – sample volume (mL), m_s – sample mass (g). TPC results were expressed in mg GAE/g edw.

E. Statistical Analysis

The values are expressed as a means of at least 3 replicate measurements with SD. The OAP was assessed by using integrated value, the so-called ‘antioxidant score’: (1) for extracts (ASE), which is the sum of values for the fraction obtained with the same solvent in all assays, expressed in the so-called ‘comparative integrated units’ (ciu/g edw); (2) for the whole mushroom dry material, expressed in ciu/g mdw.²³ The latter, as an ‘antioxidant score of mushroom’ (ASM), takes into account ASE and extract yields (EY) and is calculated as follows: $ASE_c \times EY_c/100 + ASE_d \times EY_d/100 + ASE_m \times EY_m/100$. The integrated values reflect the OAP of the mushroom species, which considers both extract yields and effectiveness of different polarity solvents. The concept of antioxidant scores integrates a large number of antioxidant activity data, which were obtained in this study.

III. RESULTS AND DISCUSSION

A. Extraction Yield

Efficient extraction of antioxidants requires different polarity solvents: certain antioxidants are better soluble in polar methanol and water while cyclohexane or dichloromethane are preferable for lipophilic compounds. Two approaches are applied for exhausting isolation of various components from biomaterials, namely parallel extraction with different solvents or sequential fractionation with increasing polarity solvents. The latter approach was applied in our study: non-polar cyclohexane was followed by polar aprotic solvent dichloromethane; the residues were further extracted with polar protic solvent methanol. It is obvious (Table 2) that the selected mushroom species are composed of different classes of substances from the point of view of their solubility. The yield of cyclohexane-soluble fraction was from 0.61% (*S.halopia*) to 5.14% (*R.badia*), dichloromethane yielded from 0.52% (*G.ammophilus*) to 3.42% (*T.caligatum*), methanol from 6.77% (*S.halopia*) to 41.29% (*A.pseudopratis*). The highest total yield of all fractions was obtained from *A.pseudopratis* (46.01%), while the lowest one from *S.halopia* (8.54%). The protic methanol possessing high dielectric constant gave a remarkably higher extract yields comparing with non-polar and aprotic solvents, indicating that the tested mushroom species contain low amounts of lipophilic constituents. The yields obtained by different solvents are important characteristics in applying the biorefining concept to biomaterials for their effective, preferably no-waste conversion into the fractions. For comparison, the previously reported yield of methanol extracts of *B.lupinus* was 37.67%¹²; thus, it is similar to the sum of extracts (36.01%) obtained in our study for this species; however, in the reported study the initial material was extracted. Sequential extraction of *A.devoniensis* with dichloromethane, methanol and water at room temperature yielded 5.3, 11.5, and 18.3%, respectively.⁹ In our study, the dichloromethane yield was lower (1.37%), while methanol yield (33.01%) which is remarkably higher. The yields of ethanolic and water-soluble polysaccharide fractions of

C. odora were 1.26 and 18.68%¹⁹; thus, the sum (19.94%) was remarkably lower than in our study (35.43%).

B. Antioxidant Potential of Different Mushroom Species

A large number of assays are used for the *in vitro* evaluation of antioxidant capacity. Since the antioxidant capacity of biological extracts is determined by a mixture of constituents, acting by different mechanisms and sometimes possess synergistic/antagonistic effects, the reliability of the evaluation of antioxidant potential increases by applying several assays.²⁴ Antioxidants can be ranked as primary or long-term antioxidants, which are active radical scavengers or hydrogen donors or chain reaction breakers, and as secondary or processing antioxidants, which are peroxide degeneraters.²⁵

ABTS⁺ and DPPH[•] scavenging, FRAP, ORAC and TPC assays are the most common *in vitro* methods. ORAC, TPC measured with Folin-Ciocalteu reagent and one of the single electron/hydrogen transfer assays (SET/HAT) were recommended for the representative evaluation of antioxidant properties.²⁶ DPPH[•]-scavenging is mainly attributed to SET; however, quenching of DPPH[•] to form DPPH-H is also possible. Other SET methods include ABTS⁺ decolourisation, FRAP and TPC assays. ORAC assay evaluates radical chain breaking antioxidant activity via HAT and measures antioxidant inhibition induced by peroxy radical oxidation. Following the recommendation of Huang et al.,²⁶ these methods were applied in our study for mushrooms for the first time. In addition, there is no available data about the tested species, except for *A. devoniensis*, *B. impolitus* and *C. odora*.

To obtain comparable results, ABTS⁺, DPPH[•], FRAP and ORAC values were expressed in TE (Trolox is a hydrosoluble analogue of vitamin E), while TPC in GAE (Table 2). A remarkable diversity in antioxidant capacity, depending on mushroom species, extraction solvent and assay method, may be observed. Therefore, the effects of these factors will be discussed in separate sections. For easier assessment of the OAP, the values were summarized using ASE and ASM, expressed in μg .

1. Antioxidant Capacity Differences between Mushroom Species

The highest antioxidant potential was demonstrated in *A. coniferarum*, *A. menieri* and *C. infractus*, particularly in SET assays, while the extracts of *L. vinosus* and *A. ovoidea* were the weakest antioxidants. For instance, the sum of TEAC (all values in $\mu\text{M TE/g}$) of *A. coniferarum* extracts in ABTS⁺ assay (727) was more than 24 times higher compared to *L. vinosus* (29.30). These differences were less remarkable in other SET assays, while ORAC varied from 35.98 (*S. halopia*) to 396 (*C. infractus*); TPC was from 15.17 (*A. devoniensis*) to 38.18 mg GAE/g (*C. infractus*). However, extract yields were dependent both on mushroom species and extraction solvent; therefore, extract TPC was recalculated for mdw, taking into account how much of the TPC is extracted with each solvent (Fig. 1): they were from 0.51 (*S. halopia*) to 5.72 mg GAE/g mdw (*C. infractus*). In general, TPC in most cases correlated with antioxidant capacity.

Taking into account all the measured characteristics, the ASMs of mushrooms (in $\mu\text{g}/\text{g mdw}$) may be grouped into four classes (Fig. 2); 0-100 (7 species); 100-200 (10 species); 200-300 (6 species), >300 (2 species). Several Agaricaceae family species (*A. freirei*, *A. pseudopratis*, *A. devoniensis*) with the scores of 233-328 possessed superior antioxidant potential compared with the majority of other dune mushrooms. The highest potential (481) had *C. infractus*; the Boletaceae family demonstrated higher scores (140-253) than Tricholomataceae (67-224).

The RSC of *A. devoniensis* methanol extract was reported to increase with higher concentration⁹; ethanol and water-soluble polysaccharide fraction of *C. odora* was weaker in DPPH[•] and OH[•] scavenging and possessed lower reducing properties comparing with the other 4 commercial mushroom species¹⁹;

however, in the mentioned studies, antioxidant indicators were expressed in percentage of scavenged radicals and therefore are difficult to compare with our results. The more effective mushroom species were reported to contain higher amounts of secondary metabolites, such as phenolics exerting multiple biological effects including antioxidant activity.²⁷

2. Effect of Extraction Solvent

Polar solvents are most frequently used for extracting polyphenolics; however, some fungal materials may also contain lipophilic compounds such as tocopherols, carotenoids and/or terpenoids; therefore, the use of different polarity solvents may provide more comprehensive information on their antioxidant potential. Our results demonstrate (Table 2) that the distribution of antioxidant activity in the fractions was highly dependent on mushroom species. The methanol extracts of *C.infractus* and *A.coniferarum* (the species possessing the highest antioxidant potential) were stronger antioxidants than their cyclohexane and dichloromethane extracts in the applied assays (except for ORAC), while for other species the results are more complicated. For instance, cyclohexane extracts of 10 mushroom species were 10-50% stronger antioxidants in SET and/or HAT assays than dichloromethane or methanol extracts (*A.freirei*, *A.devoniensis*, *A.fissuratus*, *S.subvolvatus*, *G.ammophilus*, *B.lepidus*, *B. lupinus*, *S.halopia*, *T.focale*, *C.odora*), while the results for the other 15 species were similar and the activity increased when protic solvent was applied. It is interesting to note that dichloromethane fractions of *A.menieri*, *A.pseudopratensis*, *B.luridus*, *T.caligatum*, possessed up to 40% higher antioxidant activity than methanol fractions in FRAP, while TPC was higher for methanol extracts. ORAC of dichloromethane extracts of *A.menieri*, *A.freirei*, *L.subolivaceus*, *B.lupinus*, *T.auratum*, *L.vinosus* was by 10-50% higher than those of methanol extracts, indicating that dichloromethane may be an effective solvent for antioxidants of some mushroom species.

Methanol was a good solvent for extracting antioxidants from some mushroom species. For instance, methanol extracts of the majority of species were strongest in ABTS^{•+}, DPPH[•] and FRAP assays; in addition, the yields were remarkably higher compared with other solvents. In general, cyclohexane and dichloromethane extracts were several times weaker radical scavengers than methanol extracts, except for 2 species, *A.menieri* and *T.caligatum*. Previously performed studies reported that polar solvents extract more antioxidants from botanicals than lower polarity solvents²⁸.

Integrated antioxidant potential scores (IAPS) were also calculated for the extracts isolated with different solvents for comparative assessment of the wholesome effectiveness of each solvent for the tested mushroom species. According to the score levels (expressed in $\mu\text{M TE/g}$) the fractions may be classified into 5 groups (Fig. 3B). However, it should be noted that in this case the scores calculated by summing the values measured for 1 g edw (Fig. 3A) are not similar to the scores calculated for 1 g mdw (Fig. 2).

3. Effects of Assay Method

Antioxidant activity values obtained by different assays are in a very wide range, depending on mushroom species and the solvent applied. Generally, the highest TEAC values were in the FRAP assay; their sum from all fractions (in $\mu\text{M TE/g}$) were from 128 (*M.cinereifolia*) to 905 (*C.infractus*). In the ABTS^{•+} assay they were from 29.3 (*L.vinosus*) to 727 (*A.coniferarum*); in DPPH[•] from 71.08 (*L.subolivaceus*) to 488 (*C.infractus*), while the lowest values were measured in the ORAC assay, from 35.98 (*S.halophila*) to 396 (*C.infractus*). The differences may be explained by the peculiarities of the reaction mechanisms in each assay, which depend on reaction media, pH, the structure of antioxidants involved and their interactions.

For instance, Agaricaceae, Boletaceae and Cortinariaceae species possessed high RSC in the ABTS^{•+} assay; TEAC from 164.0 (*S.subvolvatus*) to 666.0 $\mu\text{M TE/g}$ (*A.menieri*), while species from the Tricholomataceae family demonstrated lower TEAC, 128.0–580.0 $\mu\text{M TE/g}$ (Table 2). TEAC of methanol fractions, which were strong ABTS^{•+} scavengers, reported for five edible wild Australian mushroom species were in the same range, as for several species examined in our study.²⁹

The overall DPPH[•] scavenging values for Boletaceae, Agaricaceae and Tricholomataceae were in the ranges of 139-280, 71.08-264.0 and 44.4-236.0 $\mu\text{M TE/g}$, respectively. The highest overall DPPH[•] RSC was recorded with *C.infractus*, 488.0 $\mu\text{M TE/g}$. Antioxidant properties of *A.devonienis* extracts sequentially isolated by dichloromethane, methanol and water were reported previously; the methanol fraction depending on extract concentration, inhibited 3.3-65.5% (ascorbic acid 48.8-97.2%).⁹ The DPPH[•] RSC of *B.impolitus* methanol extract, expressed in effective concentration EC₅₀ was 5.81±0.17 mg/mL¹¹; in our study RSC of methanol fraction of the same species was 79.5 $\mu\text{M TE/g}$. The water-soluble polysaccharidic fraction of *C.odora* was a stronger antioxidant compared to ethanol extract in the DPPH[•] assay; this fraction revealed a higher activity in reducing power and the β -carotene bleaching inhibition assays.¹⁹ In general, the extracts isolated with polar solvent demonstrated higher antioxidant capacity in the DPPH[•] assay; however dichloromethane fractions of some species (*A.menieri*, *L.vinosus*) were remarkably stronger antioxidants in this assay compared to methanol extracts.

In the FRAP assay, the highest OAP (in $\mu\text{M TE/g}$) was determined for *C.infractum* (905), *B.luridus* (848), *A.coniferarum* (828), and *T.caligatum* (826). FRAP for other species from the same families was in the range of 367 (*A.menieri*) – 724 (*T.auratum*), whereas *M.cinereifolia* (128), *T.focale* (186) and *S.subvolvatus* (297) showed a lower reducing power. FRAP of *B.impolitus*¹¹ and *C.odora*¹⁹ methanol extracts was reported previously, however, the results were expressed in relative units. Cyclohexane or dichloromethane fractions of several mushroom species, e.g. *A.coniferarum*, *T.caligatum*, demonstrated a higher FRAP than their polar methanol fraction. For instance, dichloromethane fractions of *A.menieri* and *A.pseudopratensis* were by 20-30% more efficient antioxidants than their methanol fraction. Chelating agents may act as secondary antioxidants by reducing redox potential and stabilizing the oxidised forms of metal ions.³⁰

Finally, mushroom's antioxidant activity was evaluated using the ORAC assay, which is more related to the biological system.²⁶ The highest overall ORAC values (in $\mu\text{MTE/g}$) were found for Cortinariaceae (257.0-396.0), Gyroporaceae (339.0) and Boletaceae (331.0-363.0). For instance, a strong peroxy radical inhibitor was *B.lupinus* cyclohexane fraction (204.0), whereas the ORAC values of dichloromethane and methanol fractions of the same species was remarkably lower, 74.3 and 60.2 $\mu\text{M TE/g}$, respectively. Other Boletaceae species possessed similar ORAC; however, the methanol extracts demonstrated higher values. Some Agaricaceae species also demonstrated high ORAC (in $\mu\text{M TE/g}$), e.g. *A.freirei* (394.0), *A.pseudopratensis* (306.0), while the activity of other Agaricaceae species was 5–10-fold lower: *A.fissuratus*, 36.8; *L.subolivaceus*, 71.9. Tricholomataceae species possessed approximately 2-fold lower ORAC, *T.focale*, 137.0; *M.cinereifolia*, 226.0 $\mu\text{M TE/g}$, whereas the species of Amanitaceae, Strophariaceae, and Russulaceae demonstrated 20–50-fold lower ORAC compared to the most active mushroom species in this assay. To the best of our knowledge, ORAC of dune mushrooms has not been reported previously.

The values measured with Folin–Ciocalteu reagent and expressed in GAE or an other phenolic compound are generally accepted as representing the total phenolic content (TPC) although it is not fully correct: reaction proceeds not only with phenolics but with other reducing ability possessing compounds.²⁵ Consequently the term TPC may be used rather conditionally. Thus, the highest overall TPC value was found for *C.infractus* (Cortinariaceae), 38.2 mg GAE/g edw; for Tricholomataceae, Agaricaceae and Boletaceae they were in the ranges of 15.8-30.7, 15.2-25.3 and 16.9-26.0 GAE/g edw,

respectively. It is interesting to note that TPC of the dichloromethane fractions of *T.auratum* and *T.caligatum* were by 40% and 25% higher than the TPC of methanol or cyclohexane fractions, respectively. Dichloromethane fractions of other Agaricaceae species also contained higher TPC than other extracts, except for *A.coniferarum* and *A.devoniensis*, when methanol extracts were superior in TPC values. Previously reported TPC values of methanol extract of *B.impolitus* was 15.50 ± 0.53 GAE/g edw¹¹; water/ethanol (9:1) extract of *C.odora* 5.69 GAE/g edw.³¹ Overall the TPC values determined for these species in our study were higher, 18.4 and 30.5 GAE/g edw, respectively. It should be noted that the extracts in a previous study was obtained from the whole material, while in our study; methanol was used for re-extracting the residue after cyclohexane and dichloromethane extraction.

CONCLUSIONS

The present study has been focused on the antioxidant potential of 25 coastal dune mushroom species from the South-western region (France). The antioxidant potential of the studied mushrooms was found to be in a very wide range: differences were observed between the tested species as well as between the fractions isolated by different solvents. An assay method was also an important factor in determining antioxidant properties of mushroom extracts. *C.infractus*, *A.coniferarum*, *A.menieri*, *A.freirei* species demonstrated the highest antioxidant potential in almost all tested assays. The correlation between the total phenolics and total antioxidant activity observed in our study was not as strong as may have been expected, most likely due to the presence of various compound classes participating in the applied assays by different mechanisms. Remarkable differences in the antioxidant properties of extracts isolated with different polarity solvents also indicate the presence of a high variety of 'mycochemicals' (valuable active components in mushroom) in the tested species. Considering that a comparatively low number of compounds have been identified in the selected mushroom species until now, the results may foster further studies of mushrooms aimed at searching of new bioactive compounds, which might be of interest for various applications, such as ingredients of functional foods, nutraceuticals, pharmaceuticals and cosmetics. In general, the comprehensive evaluation of antioxidant potential of dune mushrooms indicates that *C.infractus*, *A.coniferarum*, *A.menieri*, *A.freirei* may be promising sources of antioxidants and other bioactive compounds that should be further valorized in terms of their nutritional and medicinal potential and applications.

ACKNOWLEDGMENTS

The authors are grateful for mycologist Mr. Jacques Guibertau and The Research Unit "Mycology and Food Safety" at the INRA Research Centre of Bordeaux-Aquitaine for the mushrooms samples. This study was supported by The Research Council of Lithuania (SVE-06/11) and by The Midi-Pyrénées Regional Council and SMI (International mobility support) program of INP Toulouse. It was performed in the framework of COST Action TD1203.

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Figure legends

Figure 1. Total content of phenolic compounds (TPC) in mushrooms

Figure 2. Antioxidant scores of mushrooms (ASM) integrating antioxidant activity values in the ABTS^{•+}, DPPH[•], FRAP and ORAC assays

Figure 3. Antioxidant scores of extracts (ASE): (A) integrating antioxidant activity values in the ABTS^{•+}, DPPH[•], RAP and ORAC assays; (B) according to score levels and solvent polarity expressed in percentage.

RESUME ÉTENDU EN FRANCAIS

INTRODUCTION GENERALE

En raison de la diversité métabolique, la variété écologique, rôle important dans la nature et web complexe de cycles de vie, les champignons ont attiré l'attention de divers scientifiques, mycologues, chimistes, biologistes et pharmaciens de multiples façons (Tkacz, 2004). Le niveau de consommation de champignons a augmenté au cours des dernières années, et ils restent une des sources commercialement importantes pour les producteurs d'aliments de spécialité (Nowacka et al., 2014). L'expertise scientifique est de plus en plus appliquée pour aider à atteindre les objectifs de développement primaires, qui comprennent la réduction de la pauvreté et de l'utilisation durable des ressources naturelles. De réels progrès ont été et continuent d'être réalisés dans les rôles que les champignons jouent dans la réalisation de ces objectifs (Boa, 2004).

Dans le passé, beaucoup de nouveaux métabolites secondaires ayant des structures chimiques diversifiées et différentes fonctions de bioactivité intéressantes ont été isolés à partir de champignons supérieurs. Ces molécules sont susceptibles de fournir des composés de plomb pour les nouvelles substances qui peuvent améliorer les fonctions du corps biologiques ou découvertes médicinales, comme des agents de chimioprévention possédant la bioactivité des immunomodulateurs, anticancéreux, etc. Cependant, de nombreux défis de molécules bioactives de champignons sont rencontrés y compris bioséparation, identification, le métabolisme ou le modèle de dépistage des problèmes biosynthétiques. Les chercheurs ont constamment cherché de nouvelles substances actives de champignons, cependant, les produits commerciaux comme encore sont très limitées et principalement de la culture sur le terrain, ce qui est évidemment un temps et un processus de main-d'œuvre (Zhong et Xiao, 2009). Les stratégies visant à améliorer de nouveaux métabolites secondaires produits naturels comme utiles existant dans les espèces de champignons sauvages, inconnus et underinvestigated, en raison de la prévalence élevée pourrait être une source complète de composants précieux pour industry. En outre, parmi les espèces connues de la quantité relative de champignons bien étudiés est très faible. Ce fait ainsi que les informations sur le grand potentiel des champignons microscopiques pour la production de métabolites bioactifs, l'expérience dans l'utilisation ethnomédicales de champignons, la nécessité écologique pour les champignons pour produire des métabolites secondaires bioactifs et les possibilités améliorées pour le plomb génétique, pharmacologique et analyse chimique nous faire croire que les champignons ont un grand potentiel pour la bioprospection réussie (Lindequist, Niedermeyer et Julich, 2005). Un grand, inexploitée, et chimiquement ressource diversité des métabolites bioactifs de macrochampignons sauvages offrent des possibilités de trouver de nouvelles structures de plomb pour les médicaments, la nutrition, fonctionnelle, thérapeutique et de la chimie phytochimique, et une nouvelle ère de champignons supérieurs recherche de métabolite secondaire a émergé de plus en plus (Quang, Hashimoto et Asakawa, 2006)

Selon les connaissances de mycologue folk les champignons sauvages moins connus cultivés dans la région Midi-Pyrénées de la France pourrait être une bonne

source de constituants bioactifs. Les enquêtes sur les informations rares sur la présence de métabolites secondaires naturels et leurs propriétés telles que des antioxydants, des antimicrobiens et d'autres aliments protéger et / ou promotion de la santé des composés sur les espèces de champignons sauvages sélectionnés n'a été réalisée jusqu'à présent. Sur cette base, l'hypothèse principale affirme que les espèces de champignons sauvages peuvent contenir des molécules bioactives naturelles précieuses avec des propriétés multifonctionnelles bénéfiques pour diverses applications alimentaires et médicinales, comme les ingrédients d'aliments fonctionnels et de nutraceutiques. Pendant ce temps, l'hypothèse doit être étudiée pour trouver des sources prometteuses de molécules bioactives naturelles, mener des enquêtes de détail dans celles montrant le potentiel et l'optimisation des techniques de détection dans les études biochimiques vers la sécurité des sources nouvellement découvertes, et la biodisponibilité des molécules actives.

Objectif de la thèse

L'objectif principal de cette étude était d'étudier antioxydantes, bioactivités antimicrobiens et autres des espèces de champignons sauvages underinvestigated sélectionnées pour obtenir des informations scientifiques, ce qui est nécessaire pour leur valorisation en tant que source pour la nutrition humaine et la production d'ingrédients fonctionnels.

Objectifs de notre étude:

1. Pour fractionnement matériau de champignons séchés en utilisant de plus en plus des solvants de polarité et de déterminer les rendements d'extraction.
2. Pour évaluer l'activité anti-oxydante des fractions de champignons à l'aide de plusieurs essais in vitro.
3. Pour évaluer l'activité antimicrobienne des fractions de champignons à l'aide de divers micro-organismes.
4. Pour évaluer les propriétés de cytotoxicité des espèces de champignons sauvages sélectionnées.
5. Pour isoler et purifier les métabolites secondaires des espèces de champignons sélectionnés à l'aide de diverses méthodes chromatographiques.
6. Pour élucider les structures des métabolites purifiés en utilisant des techniques spectroscopiques telles que ESI-MS, ESI-MS / MS, RMN 1H, RMN 13C, HMBC, COSY, HSQC.
7. Pour évaluer l'activité antioxydante et la cytotoxicité des principaux métabolites secondaires, isolés à partir d'espèces de champignons sélectionnés.
8. Pour examiner l'influence des extraits de champignons sélectionnés recueillis à différentes périodes de récolte de champignons et leurs composés isolés inhibitrices propriétés de l'activité contre les nématodes gastro-intestinaux in vitro.

Nouveautés scientifiques

La nouveauté scientifique de la recherche effectuée peuvent être résumées par les points suivants:

- L'évaluation systématique et complète des propriétés antioxydantes, antimicrobiennes et cytotoxiques de diverses espèces de champignons de la région Midi-Pyrénées de la France a été réalisée pour la première fois;
- Certains composés isolés à partir des espèces de champignons analysés ont été signalés pour la première fois;
- Les effets identifiés dans certains champignons espèces composés contre les nématodes gastro-intestinaux, ainsi que leurs propriétés antioxydantes et cytotoxique activité, ont été évalués pour la première fois;
- Les extraits d'espèces de champignons sélectionnés ont été signalés comme agents anthelminthiques pour la première fois;

Valorisation des travaux

Exploration plus large des bioressources étudiées, y compris diverses espèces de champignons, exige des preuves scientifiques solides sur leur composition et les propriétés. De ce point de vue d'ensemble et une évaluation systématique des antioxydants, des antimicrobiens et d'autres propriétés des espèces sauvages de champignons Midi-Pyrénées peut être considérée comme une étape importante pour la valorisation pratique de ces espèces comme une source d'ingrédients fonctionnels naturels à appliquer dans les nutraceutiques, des aliments fonctionnels et d'autres applications.

Plus précisément, les nouvelles connaissances obtenues sur hispidin, hispolon et de l'ester méthylique de l'acide inonic, les composés qui ont été identifiés dans des extraits de champignons, et qui ont été caractérisés pour leur activité cytotoxique et antioxydant, peut être utile pour les industries à la recherche de nouvelles structures pratiques pour être incorporé dans les aliments, nutraceutiques, pharmaceutiques et cosmétiques comme efficaces ingrédients naturels bioactifs.

Enfin, les résultats d'essais in vitro ont confirmé l'activité potentielle d'espèces de champignons sélectionnés et les composés isolés qui inhibent les nématodes gastro-intestinaux. Cette connaissance peut être appliquée pour développer de nouvelles préparations efficaces possédant des activités anthelminthiques pour augmenter la résistance aux populations de vers chez les ruminants. Par exemple, des extraits de champignons pourrait être un candidat naturel prometteur comme alternative aux médicaments synthétiques causant des résidus dans les matières premières animales, ce qui est une caractéristique importante dans la production de l'élevage biologique.

Points clés de la thèse

1. Les espèces de champignons sauvages sélectionnés contiennent des constituants bioactifs de valeur, tels que des antioxydants, des antimicrobiens et des agents cytotoxiques, tandis que la sélection appropriée des solvants permet aux extraits d'être obtenus avec la plus forte concentration de composés bioactifs qui pourraient être une source précieuse de produits naturels pour diverses applications.

2. Les champignons genre *P. schweinitzii* et *I. hispidus* sélectionnés ont le potentiel de l'activité *in vitro* contre les nématodes gastro-intestinaux; Toutefois, en fonction de la période de récolte de champignons, l'activité anthelminthique peut changer en termes de concentrations de constituants actifs accumulés dans le genre choisi.

Structure et contenu de la thèse

La thèse est rédigée en anglais. Il se compose d'accusés de réception, une liste d'abréviations, introduction, revue de la littérature, les matériaux et les méthodes, les résultats et la discussion, les conclusions, une liste de références, annexes, et une liste des publications pertinentes aux résultats de thèse. La thèse a 138 pages au total, les résultats présentés dans 9 tableaux et 41 figures. La liste des références comprend 244 sources bibliographiques.

Approbation des travaux

2 articles scientifiques ont été publiés sur la base de la matière de cette thèse: la revue "IJMM-Indian Journal of Medical Microbiology" (accepté) et "Science LWT-Food et de la technologie» dans la base de données d'information scientifique "Web Thomson Reuters des connaissances ». Le matériau de la thèse a été présentée dans 8 conférences scientifiques.

2. MATERIELS ET METHODES

2.1. MATERIEL FONGIQUE

En 2001, la cueillette des champignons dans le Sud de la France a concerné vingt-quatre espèces côtières dits champignons de dunes. En 2009, la cueillette dans la région de Midi-Pyrénées a permis de collecter six types de champignons sauvages (*Phaeolus schweinitzii*, *polypore hérissé*, *columbetta Tricholoma*, *Tricholoma caligatum*, *Xerocomus chrysenteron*, *Hydnellum ferrugineum*). En outre les champignons *Phaeolus schweinitzii* ont été également recueillis en 2011 et 2012. L'identification taxonomique a été faite au Centre INRA Bordeaux-Aquitaine

Station de Recherche "Mycologie et sécurité des aliments, à l'Université de Toulouse (Faculté de pharmacie) et par la Société de Mycologie de Toulouse. Tous les champignons ont été lyophilisés puis stockés dans des sacs en papier dans un dessiccateur à température ambiante (<30 ° C).

2.2 MÉTHODOLOGIE

Les champignons ont été soumis à un fractionnement en utilisant des solvants de polarité croissante (qualité HPLC) cyclohexane, le dichlorométhane, le méthanol et l'eau déminéralisée. Une extraction au Soxhlet fractionnement cohérent à partir des composés de champignon solubles dans chaque solvant a été séché dans un évaporateur rotatif et le résidu a été extrait avec de l'eau bouillant (Fig. 1). L'évaluation systématique des espèces sélectionnées de champignons antioxydant, antimicrobienne et l'activité biologique. Des extraits de champignons activité antioxydante a été abondamment évalués en utilisant des systèmes in vitro. la capacité de piégeage des radicaux libres en utilisant le 2,2-diphényl-1-pikrilhidrazilo (DPPH[•]) et un radical 3-éthylbenz-thiazol-6-sulfonique, le 2,2-bis-azine acide (ABTS^{•+}) un radical cation des procédés de décoloration. On a également trouvé des ions ferriques (Fe³⁺) est capable de procédé de réduction de la FRAP et la capacité de piégeage des radicaux libres d'oxygène (ORAC). des composés phénoliques générale (BFJK) déterminée par la méthode de Folin-Ciocalteu, et exprimées en mg d'équivalents d'acide gallique (GRE) / g d'extrait sec. indicateurs Antioxydant ont été résumées en utilisant les valeurs totales - extrait, partition antioxydant (EAB), qui est égale à la même solvant du tout une tentative pour obtenir la somme des fractions. EAB est exprimée en soi-disant «unités comparables à la somme de 1 g de poids extrait sec (PSV / g ESM). Afin d'évaluer le total EAB et extraire le rendement effet (AE) a été introduit en «score champignon antioxydant (GAC), qui est exprimée en 1 g de poids sec du champignon (PSV / g GSM). Et des extraits de champignons activité antimicrobienne a été évaluée avec des bactéries Gram-positives (*Bacillus cereus*, *Staphylococcus aureus*) et Gram négatif (*Pseudomonas aeruginosa*, *Escherichia coli*), les méthodes de diffusion des bactéries et bioautografijos disque. L'activité biologique a été étudiée en utilisant *Leishmania infantum* parasite amastigotus.

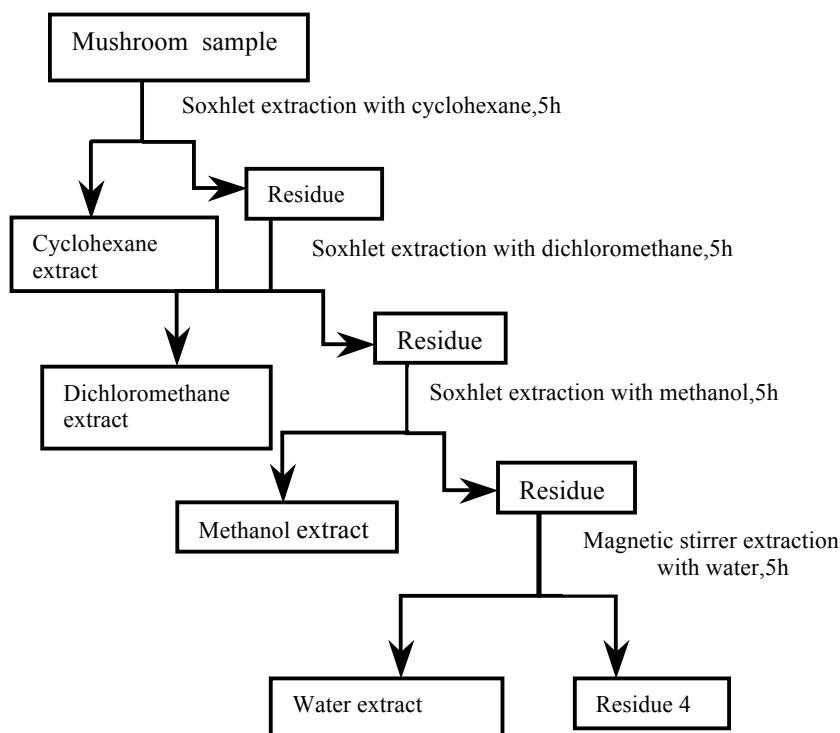


Fig. 1 Mushroom extraction scheme with Soxhlet extractor

L'évaluation des champignons sauvages fractions antioxydante, activité antimicrobienne et biologique des méthodes *in vitro* comme un objet de recherche sélectionné *M. schweinitzii* un extrait méthanolique marqué par une activité significative contre les extraits testés sur les espèces de champignons. métabolites secondaires de *M. distingués* en utilisant des techniques chromatographiques et d'explorer la spectroscopie (spectroscopie de masse (MS), spectrométrie de masse tandem (MS/MS), la spectroscopie par résonance magnétique nucléaire (RMN), la spectroscopie par résonance magnétique nucléaire du proton (^1H RMN) hétérobanduoline corrélée (HMBC, COSY, HMQC).

Isole composés activité antioxydante de l'ABTS^{•+}, DPPH[•], FRAP, méthodes ORAC. biologique des composés évalués génotoxicité des procédés *in vitro* sur des lymphocytes humains et des micronoyaux chromosomiques entraînant des écarts par rapport lymphocytes normaux, ainsi que pour mener à bien les dommages et les mutations de l'ADN initial dans la recherche sur les cellules bactériennes. activité antiparasitaire Enquête de métabolites secondaires à l'aide de *L. infantum* promastigotes.

Mushroom, qui était un choix entre les différentes périodes de l'année, les espèces et leurs composés isolés activité anthelminthique a été déterminée en utilisant un nématodes gastro-intestinaux *H. Contor* et *T. culumbiformis*.

Les expériences ont été répétées au moins 3 fois, les valeurs moyennes et leurs écarts types pour calculer le programme MS Excel 2003. L'examen des propriétés antimicrobiennes, la cytotoxicité et l'activité biologique des extraits a été évaluée et le solvant a mené extraction influence.

3. RÉSULTATS ET DISCUSSION

3.1. PROPRIETES ANTIOXYDANTES DES CHAMPIGNONS SELECTIONNES

Pour obtenir des valeurs comparables les résultats de l'ABTS^{•+} DPPH[•], FRAP et les dosages ORAC ont été exprimées dans le Trolox (analogue hydrosoluble de la vitamine E), les équivalents, à savoir la quantité de Trolox iM possédant une capacité antioxydante similaire à 1 g EDW, tandis que PTC a été exprimée en mg d'équivalents d'acide gallique par g de edw. diversité remarquable de la capacité antioxydante, selon les espèces de champignons, solvant d'extraction et de la méthode de dosage, peut être clairement observé. Par conséquent, il convient de discuter de manière cohérente l'effet de ces facteurs dans des sections distinctes. Pour une évaluation plus facile du potentiel antioxydant de différentes espèces de champignons, ainsi que les effets de différents solvants et des essais d'activité anti-oxydante appliquée. Les caractéristiques anti-oxydantes ont également été résumés en utilisant «scores antioxydants» des extraits (ASE) et de matériel de champignon sec (ASM) et exprimés dans les soi-disant «unités intégrées comparatifs» (CIU).

3.1.1. Rendement du fractionnement des extraits de champignons par différents solvants

Une extraction efficace des antioxydants et d'autres molécules biologiquement actives nécessite l'utilisation de solvants ayant des polarités différentes: certains antioxydants sont plus solubles dans les solvants polaires tels que le méthanol et l'eau, tandis que le cyclohexane ou le dichlorométhane sont préférables pour l'isolement des composés lipophiles. Deux approches principales peuvent être appliquées pour évacuer l'isolement des différents composants du matériel biologique, à savoir l'extraction parallèle de matériau initial avec différents solvants ou fractionnement séquentiel avec l'augmentation de la polarité et les solvants de constante diélectrique. Cette dernière approche a été appliquée dans notre étude: cyclohexane non polaire a été suivie par le dichlorométhane polaire solvant aprotique; les résidus ont ensuite été extraits avec du méthanol polaire solvant protique et le processus pour plusieurs espèces a été finalisé avec l'eau bouillante possédant la plus haute constante diélectrique. Il est évident que les champignons sélectionnés sont composés de très différentes classes de substances à partir du point de vue de leur solubilité dans les solvants appliqués. Les rendements des espèces de champignons totaux classés par familles. familles dominantes, qui possédaient des

rendements totaux significatifs, étaient Agaricaceae, Tricholomataceae et Boletaceae. Le rendement de la fraction soluble dans le cyclohexane non polaire selon les espèces de champignons était de 0,61% (*S. halophila*) à 5,14% (*R. badia*), aprotique dichlorométhane solvant a donné de 0,52% (*G. ammophilus*) à 3,42% (*T. caligatum*), le méthanol protique de 6,77% (*S. halophila*) à 41,29% (*A. pseudopratisensis*), plus d'hydrogène composants de liaison. Alors que l'eau bouillante de 6 espèces en outre extraites de 2,94% (*H. ferrugineum*) à 25,29% (*T. columbetta*) des composants hydrosolubles. Ainsi, le rendement maximal total des fractions a été obtenue à partir de *T. caligatum* (63,15%), tandis que le plus bas de *S. halophila* (8,54%). solvants protiques possédant une constante diélectrique élevée ont remarquablement élevés des rendements d'extraction par rapport aux solvants non polaires et aprotiques. Cela prouve que toutes les espèces de champignons testés contiennent de faibles quantités de constituants lipophiles. Les rendements obtenus par les différents solvants sont des caractéristiques très importantes dans l'application du concept de bioraffinerie à biomatériaux pour leur préférence sans déchets conversion effective, dans les fractions pour différentes applications.

3.1.2. Différences des capacités antioxydantes entre espèces de champignons

Le potentiel antioxydant le plus démontré *I. hispidus* et des extraits de *P. schweinitzii*, en particulier dans des essais de SET, tandis que les extraits isolés d'espèces comme *L. vinosus* et *T. columbetta* étaient les sources d'antioxydants les plus faibles de ces essais. Par exemple, la somme des TEAC d'extraits *I. hispidus* dans les essais ABTS^{•+} à balayage (3108,9 $\mu\text{M TE / g}$) était plus de 100 fois plus élevée comparée à celle de *L. vinosus* L. (29,30 $\mu\text{M TE / g}$). Ces différences étaient moins remarquables dans d'autres essais de SET, tandis que les valeurs ORAC variaient de 35,98 (*S. halopia*) à 462 $\mu\text{M TE / g}$ (*I. hispidus*), le PTC variant de 15,17 (*A. Devoniensis*) à 84,47 mg GAE / g (*I. hispidus*). Cependant, les rendements d'extraction étaient dépendants à la fois sur les espèces de champignons et du solvant d'extraction utilisé, donc des valeurs TPC obtenus pour les extraits ont été recalculés pour 1 g de champignons de poids sec (MDW), en tenant compte de la quantité de PTC extraite par chaque solvant. Les valeurs de PTC exprimées de cette manière sont présentés sur la Fig. 2: ils étaient de 0,51 mg GAE / g mdw (*S. halopia*) à 5,90 mg GAE / g mdw (*I. hispidus*). En général, les valeurs de PTC dans la plupart des cas étaient en accord avec les valeurs de la capacité antioxydante obtenus dans d'autres essais.

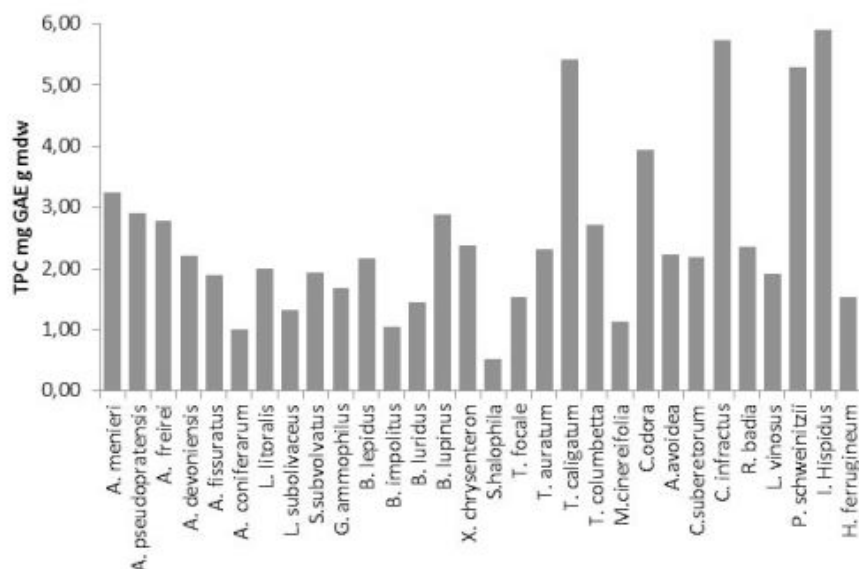


Fig. 2 Total content of phenolic compounds (TPC) in mushrooms

En général, les solvants polaires sont plus régulièrement utilisés pour l'extraction d'antioxydants à partir de matériaux contenant des composés polyphénoliques comme les principaux composés actifs anti-oxydants; Cependant, certains matériaux d'origine de champignons peuvent également contenir des composés lipophiles tels que les tocophérols, les caroténoïdes et les terpènes, et l'utilisation de différents solvants de polarité peut fournir des informations plus complètes sur leur potentiel antioxydant, en particulier dans le cas des espèces de champignons moins étudiés. Nos résultats démontrent clairement que la distribution des principes actifs anti-oxydants dans les fractions isolées avec différents solvants est fortement dépendante des espèces de champignons. Les extraits isolés à partir hispidus I et P. schweinitzii (l'espèce possédant le potentiel anti-oxydant le plus haut) avec des solvants protiques du méthanol et de l'eau étaient remarquablement antioxydants plus puissants que les extraits de cyclohexane et de dichlorométhane de la même espèce dans tous les essais, tandis que pour d'autres espèces, les résultats sont plus compliqué. Par exemple, des extraits de cyclohexane de 10 champignons espèces étaient 10-30% plus forte en antioxydants dans SET et / ou des analyses de la HAT que du dichlorométhane (A. freirei, A. Devoniensis, A. fissuratus, B. lupinus, B. Lévide, S. subvolvatus, G. ammophilus, S. halophila, T. et C. odora focale), tandis que 20 autres espèces résultats étaient similaires et l'activité a augmenté avec un solvant protique. T. columbetta ou X. chrysenteron (sauf DPPH• dosage) fractions cyclohexane dans tous les dosages testés étaient de 10 à 40% plus élevé que les fractions de dichlorométhane. Toutefois, il convient de mentionner que ces espèces sont caractérisées en tant que potentiel antioxydant suffisant. Il est intéressant de noter que les fractions de dichlorométhane d'espèces A. menieri, A.

pseudopratinensis, *B. luridus* et *T. caligatum*, possédait plus forte, l'activité antioxydante parfois 40% plus élevée que la fraction de méthanol dans FRAP, tandis que les valeurs de PTC étaient respectivement plus élevées pour ceux-ci fractions. *A. menieri*, *A. freirei*, *L. subolivaceus*, *B. lupinus*, *T. auratum* et les valeurs de Vinosus L. dans le test ORAC était 10-50% plus élevée que la fraction de méthanol et a indiqué le dichlorométhane et le cyclohexane comme solvant approprié pour extraire les phénols.

Notre étude montre que le méthanol et l'eau peuvent être des solvants utiles pour l'extraction d'antioxydants à partir de certaines espèces de champignons après l'application de solvants organiques polaires. L'eau est un solvant préférable en termes de toxicité et de la disponibilité; cependant, ne sont pas toujours suffisamment efficace pour l'isolement des composés bioactifs champignon. Par exemple, l'extrait de l'eau de *I. hispidus* est l'antioxydant le plus fort en ABTS^{•+}, FRAP et des essais de PTC; toutefois, il est presque six fois plus faible dans des essais ORAC par rapport à la fraction de méthanol. Il est également important de noter que le rendement de l'extrait de l'eau de *I. hispidus* était remarquablement plus élevé que les rendements obtenus avec d'autres solvants; fraction d'eau constitué 67% du total des extractif. Les extraits aqueux des 6 autres espèces testées étaient également nettement moins efficace dans des essais ORAC, à l'exception de *H. ferrugineum*, lorsque la différence entre le méthanol et l'eau des fractions constitué de seulement 14%. Il convient de noter que les extraits aqueux ont été obtenus en faisant bouillir les résidus d'extraction avec des solvants organiques et, dans ce cas, une certaine hydrolyse et d'autres procédés impliquant des modifications chimiques susceptibles de se produire dans le produit d'extraction. En général, les extraits de méthanol de la plupart des espèces ont été la plus forte dans ABTS^{•+}, DPPH[•] balayage, FRAP et les rendements ont été remarquablement plus élevé que les rendements obtenus avec d'autres solvants; cependant, cyclohexane et de dichlorométhane fractions ont été piègeurs de radicaux plusieurs fois plus faibles que les extraits méthanoliques polaires, à l'exception de plusieurs espèces décrites ci-dessus. De nombreuses études réalisées précédemment rapporté que les solvants polaires extraient plus d'antioxydants de plantes que des solvants de polarité inférieure (Brahmi et al., 2012), ce qui est vrai pour certaines espèces de champignons sauvages. scores potentiels antioxydants intégrés ont également été calculées pour les extraits isolés avec différents solvants pour l'évaluation comparative de l'efficacité de chaque solvant pour les espèces de champignons sauvages testés

3.2. PROPRIETES ANTIBACTERIENNES ET BIOLOGIQUES DES CHAMPIGNONS SELECTIONNES

3.2.1. Les propriétés antibactériennes des extraits de champignons

Evaluation de l'activité antioxydante des extraits isolés à partir des espèces de champignons sélectionnés par différents solvants a révélé remarquable variabilité dans les valeurs obtenues. Il est connu que de nombreux composés actifs antioxydants peuvent également posséder des effets différents contre des micro-organismes.

Une activité antimicrobienne a été considérée comme un indicateur des espèces de champignons pour produire des métabolites secondaires bioactifs d'intérêt thérapeutique potentiel. Par conséquent, l'étude a été poursuivie pour le criblage préliminaire des propriétés anti-microbiennes des extraits contre les deux bactéries Gram-positives et deux bactéries Gram-négatives. Les résultats obtenus pour les 80 extraits isolés à partir de 30 espèces de champignons. Les représentants des différents genres des différences marquées dans leur capacité à montrer des activités antimicrobiennes (Fig. 3). Les espèces du *B. impolitus*, *B. luridus*, *G. ammophilus*, *S. halophila*, *T. caligatum*, *C. infractus* ont été particulièrement productive (activité 75-95%) alors que les membres de la famille Agaricaceae, les espèces du *B. lepidus*, *B. lupinus*, *T. focale*, *T. auratum*, *C. odora*, *A. ovoidea*, *R. badia*, *L. vinosus*, *C. suberretorum*, *M. cinereifolia* a donné des résultats intermédiaires (activité de 30-50%). Par exemple, les plus grandes zones d'inhibition de plus de 15 mm a été observée pour la fraction de méthanol et de *P. I. schweinitzii hispidus* contre *P. aeruginosa* et *B. cereus*, respectivement; Aussi fort effet (> 15mm) a démontré l'extrait de méthanol de *C. infractus* avec une activité partielle contre *P. aeruginosa*. Il est intéressant de noter que *I. hispidus*, *P. schweinitzii* et *C. infractus* possédait le potentiel antioxydant le plus élevé, en comparant avec d'autres espèces étudiées, qui étaient moins actifs dans des tests antimicrobiens. Les différences entre les représentants de toutes les espèces pourraient refléter les diverses substances de ces champignons et ont deux effets: il peut avoir une activité forte, inhiber toute croissance de micro-organisme ou une activité partiellement inhibition autour du disque. Les espèces de champignons d'essai ont montré une plus grande activité antibactérienne contre les bactéries Gram-positives que des bactéries Gram-négatives. Par exemple, les bactéries testées ne sont pas inhibées 9 des fractions testées (*A. menieri* (C), *A. fissuratus* (C), *A. coniferarum* (C), (C de L.), *B. impolitus* (D) *M. cinereifolia* (D), *C. infractus* (C), *R. badia* (C), *L. vinosus* (C)). En comparant les solvants, les extraits de méthanol dans la plupart des cas ont été les plus forts agents antimicrobiens, tandis que Agaricaceae fraction famille D possédait la plus forte activité inhibitrice sauf *A. Devoniensis* et la fraction L. qui n'a pas été testé. fraction M et C de la famille Boletaceae avait une activité très similaire, à l'exception des espèces de *B. lupinus* M fraction ont montré une activité plus forte.

En comparant les espèces de champignons, on peut observer que la fraction de *B. luridus* isolé avec solvant C possédait une activité antibactérienne contre les bactéries testées dans l'essai de diffusion sur gélose et a montré plus grandes zones claires actives plus que 15mm, mais l'extrait n'a pas été efficace contre *E. coli*. Aussi fort effet partiel de *B. lepidus* (extrait de D), *B. impolitus* et *S. halophila* (extraits de M), contre *S. aureus*; *G. ammophilus* (D et M extrait) et *T. caligatum* (M extrait)

contre *B. cereus*; *B. luridus* et *C. infractus* (extraits M) contre *P. aeruginosa*; *A. pseudopratis* contre *E. coli* inhibait microorganismes dans la gélose diffusion dosage plus de 15mm testé, mais n'a pas complètement inhibé la croissance. Figure. 3. donne un aperçu du contenu des champignons des espèces avec des zones actives claires et des zones partiellement inhibition contre les microorganismes testés

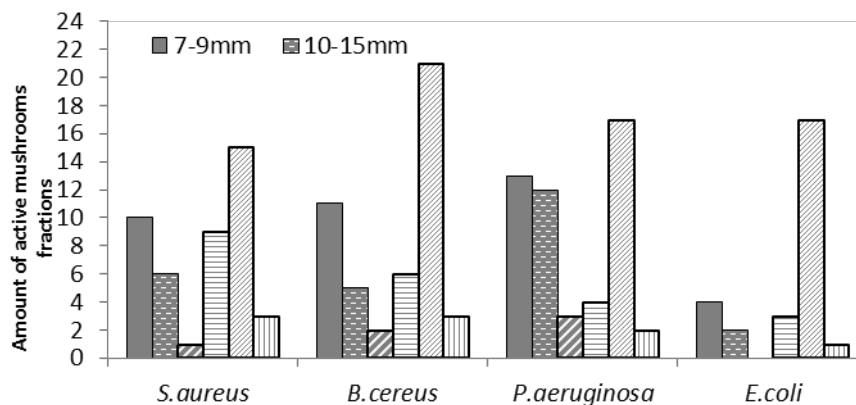


Fig. 3 Active content of mushroom fractions against tested microorganism

3.3. ISOLATION DES METABOLITES SECONDAIRES ET IDENTIFICATION DES EXTRAITS METHANOLIQUES DE PHAEOLUS SCHWEINITZII (FR.)

De l'évaluation des potentiels antioxydants et biologiques des champignons de la région Midi-Pyrénées, il a été constaté que l'extrait de méthanol fongique *P. Schweinitzii* est caractérisé par une activité significative selon les diverses méthodes étudiées. Bien que plusieurs composés ont été identifiés, il n'y a pas d'informations détaillées sur les métabolites secondaires responsables des activités biologique et antioxydante. Ces études visent à isoler les composés actifs de l'extrait de méthanol de *P. Schweinitzii* en utilisant la chromatographie préparative et méthodes d'analyse spectroscopiques (ESI-MS, MS / MS, RMN 1H, RMN 13C, HMBC, COSY, HMQC). Trois composés on pu être identifiés : hispidine, hispolone et ester méthyliques d' inonotus, les deux derniers composés ont été isolés à partir de *P. schweinitzii* et décrits pour la première fois (4, 5, Fig. 6)

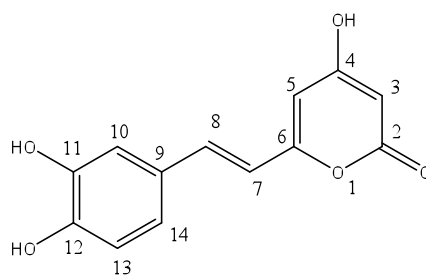


Fig. 4 Structure of hispidin (6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone)
(compound 3)

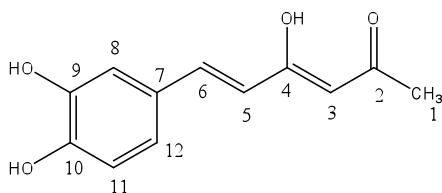


Fig. 5 Structure of hispolon (6-(3, 4-Dihydroxyphenyl)-4-hydroxyhexa-3,5-dien-2-one)

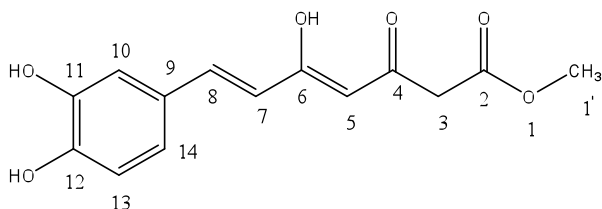


Fig. 6 Inonic acid methyl ester (7-(3,4-dihydroxy-phenyl)-5-hydroxy-3-oxo-hepta-4,6-dienoic acid methyl ester)

3.4. EVALUATION DES ACTIVITES ANTIOXYDANTES, BIOLOGIQUES ET ANTIPARASITAIRES DES COMPOSES ISOLES DE *P. SCHWEINTIZII*

L'objectif était de déterminer les activités antioxydantes et biologiques majeure des composés isolés dans différents essais mais aussi d'étudier l'influence des différentes périodes de récolte des champignons *P. schweinitzii* et *I. hispidus* sur la composition des extraits et sur les activités inhibitrices des composés isolés vis à vis des nématodes gastro-intestinaux in vitro.

3.4.1. Activité antioxydante de l'Hispidine

L'évaluation de l'activité antioxydante de hispidine isolée a été effectuée au moyen de différentes méthodes mais seuls les essais de transfert d'atome (SET ou HAT) ont été utilisés ont permis d'obtenir les résultats souhaités.

Le test ORAC utilise un principe basé sur la fluorescence (parfois abrégé en ORAC-FL) et mesure un indice antioxydant par rapport à un dérivé hydrosoluble de la vitamine E (Trolox), utilisé comme antioxydant de référence. L'exposition du fluorophore, dans ce cas, la fluorescéine (FL), aux radicaux peroxydes amène à un processus d'oxydation illustré par une décroissance de l'émission de fluorescence au

cours du temps. Dans le test ORAC la perte de fluorescence FL correspond généralement à un temps d'induction et est tributaire de la capacité antioxydante d'un composé. Les expériences ont montré un meilleur profil cinétique pour l'hispidine que pour le Trolox démontrant une stabilité à l'oxydation de protection renforcée. On a calculé que la capacité antioxydante de 1 g dans l'essai ORAC avec l'hispidine est équivalent à $2,825 \pm 0,7$ mM TE / g, ce qui équivaut à 0,706 g de Trolox.

Le test DPPH[•] est largement utilisé comme procédé chimique simple et précis pour la mesure de la SRC de diverses substances. Ceci est dû aux propriétés électroniques partagés par DPPH[•] et les radicaux peroxy (l'électron non apparié est délocalisé respectivement entre la paire d'azote ou d'oxygène), de telle sorte que la vitesse de réaction entre DPPH[•] et de nombreux antioxydants fournit une bonne approximation des activités de piégeage. On peut observer que les deux composés ont démontré des activités antioxydantes presque similaires aux concentrations appliquées: le pourcentage DPPH[•] en fonction du composé étaient dans les gammes de concentrations de 2,82 à 83,04% et de 3,32 à 87,53% pour respectivement le Trolox et l'hispidine. La valeur de CI₅₀ pour hispidin montrant sa concentration nécessaire pour réduire de 50% dans la réaction DPPH[•] a été mesurée à 3,66 mM. On a calculé que la capacité antioxydante de 1 g de hispidin dans cet essai est équivalent à $2,09 \pm 0,04$ mM TE / g, ce qui équivaut à 0,52 g de Trolox.

La méthode ABTS est basée sur la capacité de l'hispidine à inhiber (décolorer) l'ABTS^{•+} en comparaison avec un antioxydant de référence (le Trolox). La RSC ABTS^{•+} de l'hispidine et des solutions de Trolox à différentes concentrations, qui étaient de 1,57 à 73,83% et 1.20- 31.88%, respectivement. Par conséquent, dans cet essai de RSC l'hispidine était significativement plus élevée que celle de Trolox, en particulier à la concentration du composé supérieur. On a calculé que la capacité antioxydante de 1 g de hispidin dans cet essai est équivalent à $7,27 \pm 0,03$ mM TE / g, ce qui équivaut à 1,81 g de Trolox.

Le dosage Ferric Reducing Antioxidant Power (FRAP) mesure la réduction du fer ferrique (Fe³⁺) en fer ferreux (Fe²⁺) en présence d'anti-oxydants, qui sont des agents réducteurs ayant des potentiels de réduction de demi-réaction Fe³⁺ / Fe²⁺. Dans le test FRAP, l' hispidin a révélée un potentiel nettement plus fort pour réduire Fe³⁺ à Fe²⁺ que le Trolox alors que l'activité variait entre 11.70- 49.71%, et trolox 8.77- 20,18%. La valeur de la capacité antioxydante de l'hispidine exprimée en équivalents de Trolox était $15,54 \pm 0,2$ mM TE / g. La concentration inhibitrice maximale nécessaire pour réduire de 50% des radicaux Fe³⁺ est $194,37 \pm 3,28$ µM / l de hispidin.

3.4.2. Activité biologique des composés isolés de *P. schweinitzii* et *I. hispidus*

3.4.2.1. Détermination de l'activité antileishmatique et de la cytotoxicité des composés isolés

A la recherche de substances bioactives précieux isolés composés de *P. schweintzii* ont évalué l'activité anti-proliférative in vitro contre de *L. infantum* formes promastigotes. En parallèle l'influence de cytotoxicité sur une ligne de la souris a été évaluée pour hispidin, hispolon et d'ester méthylique d'acide inonotic. Amphotéricine B a été choisi comme référence médicament antileishmanienne, et doxorubicine utilisé comme témoin positif de la cytotoxicité. Toutes les molécules ont d'abord été sélectionnés in vitro sur la scène de promastigotes de *L. infantum* en déterminant leurs concentrations inhibitrices de 50% (IC50) et en les comparant à celle de l'amphotéricine B. Afin d'évaluer leur sélectivité d'action, les molécules testées ont également été évalués in vitro en ce qui concerne leur cytotoxicité mesurée par les concentrations cytotoxiques 50% (CC50) sur la lignée cellulaire de macrophages de souris J774A.1, donnant accès aux indices de sélectivité correspondants ($SI = CC50 / IC50$)

Les composés testés présentaient une activité modérée antileishmanienne IC50 dans l'ordre suivant: l'ester méthylique d'acide Inonotic <hispolon <hispidin. Ainsi, par rapport à la cytotoxicité de l'amphotéricine B ($2,47 \pm 0,24 \mu M$) ou de la pentamidine ($0,53 \pm 0,57 \mu M$) ont été composés à partir de 7-12 fois moins toxiques que le médicament mentionné et globalement légèrement toxiques. En comparant l'indice de sélectivité de médicament référencé pentamidine (1,04), les molécules ont été 33 et 51% de moins sélectifs pour hispolon et de l'ester méthylique d'acide inonotic respectivement. Pendant ce temps hispidin (25%) était plus sélective sont comparés avec le médicament mentionné pentamidine. Cependant, le composé testé avait un indice de sélectivité très faible par rapport à l'amphotéricine B ou Miltéfosine.

3.4.2.2. Evalutaion complète de l'activité biologique de l'hispidine isolée de *P. Schweintzii*

Dans cette étude, une évaluation détaillée du *P. schweintzii* hispidine isolé activité génotoxique. Des études montrent que hispidinas n'a pas été l'analyse génotoxique du test du micronoyau (dose gamme 10-150 mg / ml) et ne provoquent pas une augmentation significative des aberrations chromosomiques. Cependant, le composé isolé caractérisé études d'échange de chromatides sœurs génotoxiques et a provoqué une augmentation dose-dépendante statistiquement significative en échange de chromatides. Hispidinas n'a pas induit de mutations génétiques chez les bactéries des cellules de *Salmonella typhimurium* et n'a pas violé les lymphocytes ADN donneurs.

3.4.2.3. Détermination de l'effet vermifuge sur des nématodes gastro-intestinaux

Les composés isolés de *P. schweintzii* ont été testés comme agents anthelminthiques sur prédominant hématophage parasite *H. contortus* et souvent co-dominant dans les populations de parasites *T. colubriformis* .. Le dosage de exsheathment larvaire a été développé pour mesurer la capacité des hispidin et

hispolon retarder ou inhiber et artificielle induire le processus de exsheathment des larves L3 infectieux (Bahuaud et al., 2006). En empêchant la exsheathment composés de processus possèdent une activité de vermifuges et les larves peuvent ne pas être capables d'infecter l'animal; Les larves sont donc ensheathed excréteur dans le tractus digestif des animaux.

Hispolon n'a pas été testé sur les larves de *H. contortus*, mais a provoqué un peu retarder seulement avec une concentration de 300 pg / ml dans le taux de *T. colubriformis* de exsheathment. En 3 heures de contact hispidin a conduit à une forte inhibition des deux espèces de nématodes avec la concentration testée la plus élevée de 300 pg / ml. Les larves étaient $3,97 \pm 2,88\%$ et $16,52 \pm 7,72\%$ exsheathed après 60 minutes pour *T. colubriformis* et *H. contortus* respectivement. En revanche, pour les autres concentrations de 150 et 75 pg / ml, exsheathment a été retardée de façon significative (15-74% et 31-90% respectivement).

Le résultat de l'efficacité des processus de exsheathment larvaire dépend de hispidin et le poids moléculaire de hispolon et composés conformation structurelle. Par exemple, les effets anthelmintiques de flavan-3-ols ont montré que l'inhibition de exsheathment a été associé au nombre total de groupes hydroxyles, le poids moléculaire des molécules, et la présence du groupement OH en C anneau (Brunet et Hoste, 2006). Les variations du taux de exsheathment appartiennent également à l'interaction entre les molécules et les protéines bioactive composition des larves de nématodes.

Les présents résultats ont confirmé les propriétés vermifuges des deux *P. schweinitzii* et *I. hispidus*. Les effets des deux champignons étaient dépendants pour les nématodes testés année. Les champignons collectés en 2011 ont montré une meilleure activité, et étaient plus efficaces avec des concentrations plus faibles. Par exemple, *P. schweinitzii* (2011) ont une forte activité d'inhibition (600 et 1200 pg / ml) et on induit à moins de 20% de exsheathment pendant 60 min sur de *H. T. colubriformis* et *contorus* également des résultats similaires ont été produits par *I. hispidus* . Des résultats moins efficaces sur deux nématodes ont été exprimées par *P. schweinitzii* et *I. hispidus* recueillies en 2012, un léger retard a été démontrée à seulement la plus forte concentration et l'inhibition exsheathment varie 21-68% ou plus petites concentrations (150 et 300 pg / ml) étaient inactifs. Ces différences peuvent avoir plusieurs facteurs, tels que la composition bioactive des composants et des concentrations dans les champignons et les périodes de collecte ou de l'âge de l'espèce. Par exemple, le sel dans le sol modifie la composition flavonoïde dans les plantes (Tattini et Traversi, 2008).

Le contact avec *P. schweinitzii* (2011) extrait au cours des 3 concentrations testées (1200, 600 et 300 pg / ml), a donné lieu à une inhibition presque totale de exsheathment, qui a été trouvée avec *H. contorus*. Les effets inhibiteurs observés avec des extraits *I. hispidus* étaient moins efficaces et deux concentrations ont montré (1200 et 600 pg / ml) un léger retard. Les extraits de *I. hispidus* (2011) et *P. schweinitzii* (2011) l'activité étaient plus dépendants de l'année de collecte et ont montré l'efficacité puissante à deux concentrations testées (1200 et 600 pg / ml) avec

T. colubriformis, donc les concentrations les plus élevées ont été légèrement retardée pour les champignons collectés en 2012.

Une explication possible pourrait être que les deux parasites nématodes occupent différents endroits dans l'animal, ainsi que les conditions d'activation pour exsheathment varient entre caillette et nématodes intestinaux. Cependant, d'après les résultats, il est évident que des extraits de *P. I. schweinitzii* et *hispidus* ont entraîné des retards négligeables de exsheathment d'une manière dépendante de la dose pour les nématodes en particulier pour la collecte 2011. L'effet de bien connu extrait de sainfoin de légumineuses fourragères a été testé sur *H. contortus* et *T. colubriformis* larves L3 à des concentrations de 300, 600 et 1200 pg / ml. A la concentration / ml 1 200 ug, une inhibition presque totale de exsheathment de *H. contortus* et *T. colubriformis* a été observée (Brunet et Hoste, 2006). Dans notre étude, des résultats similaires ont été observés. Variabilité d'inhibition exsheathment entre les espèces de champignons pourrait être dû à plusieurs raisons et ne reflètent pas nécessairement une vraie différence entre les espèces de nématodes (Novobilský, Mueller-Harvey et Thamsborg, 2011). Les extraits diffèrent probablement en métabolites secondaires de contenu et peut-être avoir un effet de synergie dans les espèces entre *P. schweinitzii* et *I. hispidus*. De nombreuses activités de nématodes gastro-intestinaux de recherche a expliqué en raison des flavonoïdes et des tanins existence, en tant que composants biologiquement actifs dans les plantes (Acharya et al., 2014, Manolaraki et al., 2010, de Oliveira et al., 2011, Katiki et al., 2011, Alonso -Díaz et al., 2008). Cependant, en plus de polyphénols, d'autres classes de composés biochimiques peuvent également participer à la bioactivité et inhiber les parasites. Cela a été trouvé avec châtaignier ou de pin extraits d'arbres lors de l'utilisation du test d'inhibition de exsheathment (Azando et al., 2011). L'isolement et l'identification des composés d'activité précis est l'un des facteurs de comprendre les voies biochimiques affectées par l'exposition à des extraits de champignons. De telles molécules isolées (bioactifs hispidin, hispolon) de *P. schweinitzii* inhibent exsheathment sur les nématodes gastro-intestinaux et possédaient des propriétés anthelminthique.

4. CONCLUSIONS

1. Le fractionnement séquentiel de 30 champignons sauvages a montré que les rendements d'extraction augmentait avec la polarité du solvant d'extraction. L'eau, qui a la polarité la plus élevée, et le méthanol ont permis d'obtenir les rendements d'extraction les plus élevés: 2,94% (*H. ferrugineum*), 25,29% (*T. caligatum*), 6,77% (*S. halophila*) et 41,29% (*A. pseudopratensis*). Les rendements des extraits au cyclohexane et au dichlorométhane ont été notablement plus faibles: 0,61 (*S. halophila*), 5,14% (*R. badia*), 0,52 (*G. ammophilus*) et 3,42% (*T. caligatum*). Les familles possédant des rendements totaux significatifs étaient Agaricaceae, Tricholomataceae et Boletacea.

2. Les facteurs suivants ont eu des effets sur la capacité antioxydante des extraits de champignons mesurée par l'ABTS in vitro • +, DPPH •, FRAP, ORAC et des mesures de PTC: famille de champignons et espèces, type et polarité du solvant et méthode d'évaluation. Les plus hauts scores «antioxydants», obtenus en intégrant les différentes données d'évaluation de la capacité antioxydante et exprimés en unités intégrées comparatives par gramme de poids sec de champignons, ont été mis en évidence pour *I. hispidus* (808 ciu / g mdw), *P. schweinitzii* (712 ciu / g mdw) et *C. infractus* (481 ciu / g mdw). En général, les espèces des familles Agaricaceae et Boletaceae possédaient un potentiel antioxydant supérieur, qui était respectivement dans les gammes de 109-325 et 140-253 ciu /g mdw. La corrélation entre PTC et activité antioxydante totale observée n'a pas été aussi forte que l'on pouvait s'y attendre, très probablement du fait de la présence de différentes classes de composés chimiques participant aux essais appliqués via différents mécanismes. D'une manière générale, les extraits isolés avec des solvants polaires ont été caractérisés comme possédant une activité anti-oxydante supérieure.

3. La majorité des extraits de champignons (89% du total des échantillons testés) ont démontré une activité antimicrobienne, 59% d'entre eux sont caractérisés par une activité antimicrobienne partielle et n'inhibent pas totalement les microorganismes. Les fractions au méthanol de *P. schweinitzii*, *I. hispidus* et *C. infractus* sont les plus forts agents antimicrobiens contre les microorganismes testés dans les tests de diffusion sur disque (zones d'inhibition étaient de 10 à > 15 mm) et par la méthode de bio-autographie. Il convient de noter que les mêmes espèces ont démontré le potentiel anti-bactérien le plus élevé avec des bactéries Gram-positives; *Bacillus cereus* et *Staphylococcus aureus* étant par ailleurs plus sensibles aux extraits de champignons que les bactéries Gram-négatives, *Pseudomonas aeruginosa* et *Escherichia coli*.

4. Les extraits au cyclohexane de *G. ammophilus* ainsi que des extraits au cyclohexane et au dichlorométhane de *S. halophila* présentent une bonne activité cytotoxique contre les parasites *Leishmania infantum* amastigotes: les index de sélectivité (SI) indiquant le rapport entre la cytotoxicité et l'activité contre les parasites pour les extraits mentionnés ci-dessus ont été respectivement de 49,09, 22,28 et 22,42. Ces résultats sont prometteurs pour le développement de nouvelles préparations d'antiparasites naturels.

5. L'extrait méthanolique de *P. schweinitzii*, en tant que substance possédant la capacité antioxydante forte et une activité antimicrobienne, a été fractionné par Chromatographie en Couche Mince (CCM), Chromatographie sur Colonne (CC) et par Chromatographie en Phase Liquide à Haute Performance (HPLC). Le dosage par auto-biographie (TLC -DPPH •) a révélé 2 fractions présentant des capacités de piégeage des radicaux remarquables qui ont ensuite été purifiées par CC et HPLC.

6. Trois constituants principaux, à savoir l'hispidine, l'hispolone et l'ester méthylique de l'acide inonotique ont été identifiés dans *P. schweinitzii* en utilisant une gamme de techniques spectroscopiques - ESI-MS, ESI-MS / MS, RMN 1H, 13C RMN, HMBC, COSY, HSQC. L'Hispolone et l'ester méthylique de l'acide inonotique sont rapportés dans *P. schweinitzii* pour la première fois.

7. Les activités des principaux composants purifiés ont été testés pour leur capacité antioxydante et leur cytotoxicité. L'hispidine est un antioxydant efficace s'après les tests ABTS • +, DPPH •, FRAP et ORAC. En général, son activité est similaire à l'antioxydant synthétique de référence, le Trolox. L'évaluation in vitro de l' hispidin sur des lymphocytes humains dans le micronoyau et l'induction d'aberrations chromosomiques dans les lymphocytes humains n'a pas révélé de génotoxicité. En outre, l'hispidin n'a pas induit des dommages de l'ADN primaire et de mutations dans la cellule bactérienne. L'hispidin, l'hispolone et l'ester méthylique de l'acide inonotic font preuve d'une activité contre les promastigotes de *L. infantum* et ont eu des effets immunomodulateurs modifiant la réponse antiphrastique des macrophages infectés.

8. Les extraits de *P. schweinitzii* et *I. hispidus* sont des inhibiteurs puissants sur les larves nématodes gastro-intestinaux de *H. contortus* et *T. colubriformis*. Toutefois, les effets des extraits isolés à partir des champignons collectés dans les différentes années sont différents, ce qui indique que les composants actifs peuvent être présents à des concentrations différentes. L'hispidine a conduit à une forte inhibition avec la plus forte concentration testée (300 pg / ml) contre les deux nématodes testés, tandis que l'hispolone a provoqué un effet retardateur vis à vis de *T. colubriformis*.

9. L'évaluation générale, complète et systématique des propriétés antioxydantes et antimicrobiennes des espèces de champignons sauvages étudiés, a fournit des données importantes pour la poursuite des travaux via leur valorisation comme source d'ingrédients précieux. Plus précisément, les extraits isolés à partir des espèces possédant le potentiel antioxydant le plus fort peuvent être de prometteurs agents protecteurs contre les dommages oxydatifs et des inhibiteurs de nématodes gastro-intestinaux. Les principales composants de ces espèces, l'hispidine, l'hispolone et l' ester méthylique de l'acide inonotic peuvent représenter un groupe de nouveaux composés naturels pour le développement d' ingrédients fonctionnels pour les nutraceutiques, d' aliments fonctionnels, de produits pharmaceutiques,... D'autres recherches devraient à l'avenir se concentrer sur la compréhension des mécanismes de la performance des extraits, des fractions et des composés purifiés en augmentant le spectre des analyses in vitro (par exemple via des cultures de cellules) et en développant des études in vivo

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