UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE FARMACIA

Departamento de Nutrición y Bromatología II

TESIS DOCTORAL

Caracterización química y evaluación de la actividad biológica de setas silvestres y cultivadas comestibles

Chemical characterization and evaluation of the bioactive properties of wild and cultivated edible mushrooms

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Madrid, 2018

UNIVERSIDAD COMPLUTENSE DE MADRID
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CARACTERIZACIÓN QUÍMICA Y EVALUACIÓN DE LA ACTIVIDAD BIOLÓGICA DE SETAS SILVESTRES Y CULTIVADAS COMESTIBLES

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Directoras:
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UNIVERSIDAD COMPLUTENSE DE MADRID
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Bromatologia

Mª DOLORES TENORIO SANZ, PROFESORA TITULAR DEL ÁREA DE NUTRICIÓN Y BROMATOLOGÍA Y DIRECTORA DEL DEPARTAMENTO DE NUTRICIÓN Y BROMATOLOGÍA II: BROMATOLOGÍA, DE LA FACULTAD DE FARMACIA, DE LA UNIVERSIDAD COMPLUTENSE DE MADRID,

CERTIFICA QUE:

El presente trabajo de investigación titulado "**Caracterización química y evaluación de actividad biológica de setas silvestres y cultivadas comestibles**" se ha realizado en el Dpto. de Nutrición y Bromatología II (Facultad de Farmacia, UCM), Instituto De Investigação e Inovação em Saúde de la Universidad De Porto (i3S) y la Escola Superior Agrária (Instituto Politécnico de Bragança), bajo la dirección de las Doctoras Patricia Morales Gómez, Maria Helena da Silva de Vasconcelos Meehan y Isabel Cristina Fernandes Rodrigues Ferreira, y constituye la Memoria que presenta Filipa Sofia Dinis Reis para optar al Grado de Doctor, con mención europea.

Y para que conste, a los efectos oportunos, firmo el presente certificado en Madrid a seis de abril de dos mil diecisiete.

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CERTIFICAN QUE:

Filipa Sofia Dinis Reis, ha realizado bajo su dirección en el Dpto. de Nutrición y Bromatología II (Facultad de Farmacia, UCM), Instituto De Investigação e Inovação em Saúde de la Universidad De Porto (i3S) y la Escola Superior Agrária (Instituto Politécnico de Bragança) el trabajo de tesis doctoral que lleva por título **"Caracterización química y evaluación de la actividad biológica de setas silvestres y cultivadas comestibles"** y que constituye su Memoria de Tesis Doctoral. Dicho trabajo reúne las condiciones necesarias para su presentación y defensa para optar al grado de Doctor, con mención europea.

Y para que conste, a los efectos oportunos, firmo el presente certificado en Porto a seis de abril de dos mil diecisiete.

To my parents,

Maria Alice Almeida Dinis

António Fernando dos Santos Reis

"From the freedom to explore comes the joy of learning."

E.O. Wilson (In *The Creation: An Appeal to Save Life on Earth*, 2010)

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ABBREVIATIONS AND ACRONYMS

Abs	Absorbance
ALA	α-Linolenic acid
ANOVA	Analysis of variance
AOAC	Association of analytical communities
ATCC	American type culture collection
BrdU	Bromodeoxyuridine (5-bromo-2'-deoxyuridine)
BSIs	Bloodstream infections
Ca^{2+}	Calcium ion
CFU	Colony forming units
CH_3	Methyl group
-COOH	Carboxyl group
DAD	Diode array detector
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dubelco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenil-1-picrilhydrazil
dw	Dry weight
EC_{50}	Concentration with 50% of antioxidant activity
EDTA	Ethylenediaminetetraacetic acid
EFSA	European food safety authority
EGF	Epidermal growth factor
ESBL	Extended spectrum beta-lactamases
EU	European Union
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organization
FBS	Fetal bovine serum
FID	Flame ionization detector
FL	Fluorescence
FUFOSE	European Commission Concerted Action on Functional Food Science in Europe
fw	Fresh weight
GAE	Gallic acid equivalents
GC	Gas chromatography
GI_{50}	Concentration that inhibits 50% of cell growth

GI_{75}	Concentration that inhibits 75% of cell growth
GLUT 1 GLUT 3	Glucose transporters
GSH	Reduced glutathione
HBSS	Hank's balanced salt solution
HP	Hewlett-Packard
HPLC	High performance liquid chromatography
HSD	Honestly significant difference
$HO^{\bullet}$	Hydroxyl radical
$HO_2^{\bullet}$	Hydroperoxyl radical
H_2O_2	Hydrogen peroxide
INT	Iodonitrotetrazolium
IS	Internal standard
$L^{\bullet}$	Alkyl radical
LA	Linoleic acid
LDL	Low-density lipoprotein
$LO^{\cdot}$	Alkoxy radicals
$LOO^{\bullet}$	Lipid peroxyl radical
LOOH	Stable lipid peroxide
MA	Malt agar
MBC	Minimum bactericidal concentration
MDA	Malondialdehyde
MDA-TBA	Malodialdehyde-thiobarbituric acid chromogen
MFC	Minimum fungicidal concentration
MH	Mueller-Hinton agar
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
MUFA	Monounsaturated fatty acids
NADPH	Nicotinamide adenine dinucleotide phosphate
NCI	National Cancer Institute
nd	Not detected
$^{\bullet}NO$	Reactive nitrogen oxide
O.D.	Optical density
O_2	Singlet oxygen
$O_2^{\bullet -}$	Superoxide anion
PBS	Phosphate buffered saline

PCD	Programmed cell death
PDA	Photo-diode array detector
PFA	Paraformaldehyde
PLP2	Porcine liver primary cell culture
PSK	Polysaccharide-K
PSP	Polysaccharide-peptide
PUFA	Polyunsaturated fatty acids
RI	Refraction index
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute medium
RSS	Reactive sulphur species
SD	Standard deviation
SE	Standard error
SPSS	Statistic package for social sciences
SRB	Sulforhodamine B
SVCT1 SVCT2	Sodium-ascorbate co-transporters (SVCTs)
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
$TO^{\cdot}$	Tocopheroxyl radical
TSB	Tryptic soy broth
SFA	Saturated fatty acids
UFLC / UHPLC	Ultra-fast liquid chromatography
USA	United States of America
UV	Ultra violet
VLDL	Very low-density lipoprotein
WHO	World Health Organization
α-TTP	α-tocopherol transfer protein

INDEX

ABSTRACT

In recent years, there is a greater health care, with people increasingly looking for a healthy lifestyle and therefore healthy foods, which could also provide innovative therapeutic options. To this end, public awareness of the need for a careful and healthy diet has increased. In this way, there is a greater demand for healthy food and food products that associated with its nutritional function, have health benefits, namely functional foods, as well as food supplements and/or nutraceutics. In this context, mushrooms arise as an excellent option for their nutritional value, bioactive compounds and health-promoting properties.

Mushrooms have been pointed as valuable health foods, rich in protein, vitamins and minerals; a source of bioactive compounds, such as unsaturated fatty acids or mono/oligosaccharides; and as having bioactive properties, such as antioxidant, anti-inflammatory, or antimicrobial activities, as well as having cardiovascular beneficial effects, amog others.

Since Bragança (Portugal) is one of the regions of Europe with greater mycological biodiversity, it is important to undertake a careful identification and characterization of a large number of wild edible mushroom species. In addition, being also a region where mushroom picking is a tradition, the scientific community plays an important role in the formation of the general population, and this is also one of our research group concerns.

Thus, the present work aims to increase edible mushroom (wild and cultivated) databases, pursuing to valorize mushrooms as promising natural resources by providing new information about their potential as a source of functional ingredients and/or nutraceuticals. To this purpose, this dissertation focuses on the chemical characterization of 17 different edible mushroom species and their extracts (mainly methanolic), with subsequent evaluation of their bioactive potential, namely antioxidant, antimicrobial and antitumour properties.

The chemical characterization of the species included the evaluation of i) mushroom nutritional value (crude protein, crude fat, ash, total carbohydrates and energy) measured relying on AOAC procedures; ii) mushroom nutrient content (soluble sugars and fatty acids) assessed through chromatographic techniques, namely high performance liquid chromatography (HPLC) and gas chromatography (GC), and iii) mushroom bioactive

compounds (ascorbic acid and other organic compounds, phenolic and tocopherols) identified by HPLC or UFLC.

Generally, the studied edible mushroom species revealed low fat contents and quite low energetic values, being carbohydrates the main macronutrients present. Mannitol and trehalose were the main soluble sugars found in the studied species. Regarding the fatty acids profile, palmitic, stearic, oleic and linoleic acids were the main fatty acids quantified in all the studied species, with polyunsaturated fatty acids prevailing over saturated fatty acids. Moreover, ascorbic acid and tocopherols were also present in some species, and regarding this latter, the β- and γ- isoforms, seems to be the main isoforms presented by these natural matrices. Regarding other bioactive compounds present in the studied edible mushrooms, oxalic acid, quinic acid, malic acid, citric acid, and fumaric acid were the main organic acids detected in the studied samples; and gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, and *p*-coumaric the phenolic acids identified as well as the related compounds cinnamic acid.

The antioxidant potential of the mushroom extracts was evaluated by different *in vitro* methodologies, namely the *Folin-Ciocalteu* assay, ferricianide/Prussian blue assay, DPPH radical-scavenging assay, β-carotene/linoleate or β-carotene bleaching inhibition assay and the thiobarbituric acid reactive substances (TBARS) assay. Overall, all the studied mushrooms revealed antioxidant potential, which suggests that some of the previously compounds identified, and known for their antioxidant potential (*e.g.*, ascorbic acid, tocopherols or phenolic acids), may be related with such property.

The antimicrobial assays used different species of food contaminants, and the minimum inhibition concentration (MIC), minimum bactericidal and fungicidal concentration (MBC and MFC, respectively) were determined for the mushroom extracts. The bacteria strains included Gram-positive (*Staphylococcus aureus, Bacillus cereus*, *Micrococcus flavus* and *Listeria monocytogenes*) and Gram-negative (*Pseudomonas aeruginosa, Salmonella typhimurium, Escherichia coli* and *Enterobacter cloacae*) bacteria, and the antifungal potential was evaluated against *Aspergillus* (*A. fumigatus*, *A. versicolor*, *A. ochraceus*, and *A. niger*), *Trichoderma* (*T. víride*), and *Penicillium* (*P. funiculosum*, *P. ochrochloron* and *P. verrucosum* var. *cyclopium*) species. Among the studied species for their antimicrobial properties, some revealed very promising results (*Suillus granulatus*), since they revealed MIC's, MBC's and MFC's lower than commercially available antibiotic and antifungal drugs.

The antitumor potential was evaluated testing the mushroom extracts against a panel of human tumour cell lines (MCF-7: breast adenocarcinoma, NCI-H460: non-small cell lung cancer, HCT-15: colorectal adenocarcinoma, HeLa: cervical adenocarcinoma, HepG2 hepatocellular carcinoma, and AGS gastric adenocarcinoma), performing the sulforhodamine B (SRB) assay and calculating the percentage of cell growth inhibition (GI_{50}). Carrying out the same colorometric assay (SRB), the extracts cytotoxicity was also evaluated in non-tumour primary cell cultures obtained from porcine liver (PLP2), in order to have some preliminary results regarding their hepatotoxicity, in a cheap, fast and ethical test. In a more in-depth study (article 9), the human breast non-malignant cell line MCF-10A was used as non-tumour control. All the studied extracts revealed antiproliferative effects in the tested cell lines (with the exception of *Cordyceps militaris* in HepG2 cells) and no cytotoxicity was observed for the tested concentrations in the non-tumour cells.

Given the previously results, some in-depth studies were carried out, in order to infer the possible mechanism of action of the studied mushroom extracts. For these assays *Leccinum vulpinum* and *Ganoderma lucidum* were selected. Different techniques were carried out for both studied. *L. vulpinum* proved to decreased cellular proliferation (BrdU assay) and induced apoptosis (Western blot and flow cytometry) in breats adenocarcinoma cells (MCF-7). Moreover, the results also suggest that the extract causes cellular DNA damage (Comet assay). On the other hand, *G. lucidum* proved to induce autophagy. A gastric adenocarcinoma cell line (AGS) was transfected with a mCherry-LC3 expression vector, and tretated with the methanolic extract of *G. lucidum*. This treatment increased the formation of autophagosomes (vacuoles typical from autophagy). Moreover, the cellular levels of LC3-II were also increased, and the cellular levels of p62 decreased (Western blot), confirming that the extract affects cellular autophagy. Treatment with the extract together with lysossomal protease inhibitors (E-64d/pepstatin) caused a further increase in the cellular LC3-II levels together with an increase in p62 levels (Western blot) proving that the methanolic extract of *G. lucidum* cause an induction of autophagy rather than a reduction in the autophagic flux.

Overall, mushrooms from the Northeastern region of Portugal, proved that these natural matrix may be considered a functional food, since the compounds found therein have, besides the nutritional effect, beneficial properties such as antioxidant, antimicrobial and antitumour

potential. However, further in-depth studies such as the study of the compounds' mechanism of action both *in vitro* and *in vivo* are needed.

Since the obtained data highlight the potential of mushrooms as a source of bioactive compounds, the present study also contributes to the awareness for the conservation of the mycological resources not only for environmental issues, but also for their potential as a source of nutraceutical / pharmaceutical compounds.

RESUMEN

En los últimos años, ha incrementado el interes por cuestiones de salud publica, estilo de vida saludables y por lo tanto se la incrementado la búsqueda de alimentos saludables que además ofrezcan alternativas terapéuticas innovadoras. De esta manera, existe una mayor demanda de alimentos y productos alimenticios saludables que además de presentar un buen perfil nutricional, tengan o aporten beneficios para la salud, a saber, alimentos funcionales, así como complementos alimenticios y / o nutracéuticos. En este contexto, las setas se presentan como una excelente opción por su valor nutricional, contenido en compuestos bioactivos y propiedades beneficionas para la salud. Las setas son alimentos saludables, ricos en proteínas, vitaminas y minerales; son fuente de compuestos bioactivos, tales como ácidos grasos insaturados o mono/oligosacáridos; y que tienen propiedades bioactivas, tales como actividades antioxidantes, antiinflamatorias o antimicrobianas, además de tener efectos beneficiosos cardiovasculares, entre otros.

Bragança (Portugal) es una de las regiones de Europa con mayor biodiversidad micológica, y de gran tradición de recolección de setas silvestres, de modo que es importante realizar una cuidadosa identificación y caracterización de dicha biodiversidad, con el mayor numero de especies de setas silvestres comestibles posible.

Así, el presente trabajo tiene como objetivo principal contribuir a la construcción de bases de datos sobre setas silvestres comestibles, con el fin de revalorizarlas como recursos naturales prometedores aportando nueva información sobre su potencial como fuente de compuestos bioactivos y/o nutracéuticos. Para ello, la presente tesis doctoral se centra en la caracterización química de 17 especies diferentes de setas comestibles y sus extractos (principalmente metanólicos), para posterior evaluación de su potencial actividad biológica, en concreto de sus propiedades antioxidantes, antimicrobianas y antitumorales.

La caracterización química de la especie incluyó la evaluación de: i) el valor nutritivo de las setas (proteína bruta, grasa bruta, ceniza, carbohidratos totales y energía) mediante los procedimientos validados de la AOAC; ii) contenido de algunos nutrientes concretos en setas (azúcares solubles y ácidos grasos) evaluado mediante técnicas cromatográficas, como la cromatografía líquida de alta resolución (HPLC) y cromatografía de gases (GC) y iii)

contenido de algunos compuestos bioactivos (ácido ascórbico y otros ácidos orgánicos, ácidos fenólicos y tocoferoles) identificados por HPLC y UHPLC.

En general, las especies de setas comestibles estudiadas presentaron bajos contenidos de grasa y valores energéticos bastante bajos, siendo los hidratos de carbono el macronutriente principal. El manitol y la trehalosa fueron los principales azúcares solubles encontrados en las especies estudiadas. En cuanto al perfil de los ácidos grasos, los ácidos grasos palmítico, esteárico, oleico y linoleico fueron los principales ácidos grasos cuantificados en todas las setas estudiadas, predominando los ácidos grasos poliinsaturados frente a los ácidos grasos saturados. Por otra parte, el ácido ascórbico y los tocoferoles también estaban presentes en algunas especies, y con respecto a los tocoferoles, las isoformas β- y γ-, fueron las principales isoformas pospresentes en estas matrices naturales. En cuanto a otros compuestos bioactivos, el ácido oxálico, ácido quínico, ácido málico, ácido cítrico y ácido fumárico fueron los principales ácidos orgánicos detectados en las muestras estudiadas; mientras que el ácido gálico, ácido protocatequídico, ácido *p*-hidroxibenzoico y *p*-cumárico, fueron los ácidos fenólicos identificados así como el compuestos relacionados ácido cinámico.

El potencial antioxidante de los extractos de setas se evaluó mediante diferentes metodologías *in vitro*, como el ensayo *Folin-Ciocalteu*, el ensayo de ferricianide/Prussian blue, el ensayo de captación de radicales DPPH, el ensayo de β-caroteno/linoleato o inhibición de la decoloración del β-caroteno y el ensayo de TBARS. En general, todas las setas estudiados destacaron por su potencial antioxidante, lo que sugiere que algunos de los compuestos anteriormente identificados, y conocidos por su potencial antioxidante (por ejemplo, ácido ascórbico, tocoferoles o ácidos fenólicos), pueden estar relacionados con dicha propiedad.

Para la evaluación de la actividad antimicrobiana, se emplearon diferentes especies de contaminantes alimentarios, y se determinó la concentración mínima de inhibición (CMI), la concentración bactericida y fungicida mínima (CMB y CMF, respectivamente) para los extractos de las setas estudiadas. Las cepas bacterianas incluyeron bacterias Gram positivas (*Staphylococcus aureus*, *Bacillus cereus*, *Micrococcus flavus* y *Listeria monocytogenes*) y Gram negativas (*Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli* y *Enterobacter cloacae*) y el potencial antifúngico fue evaluado contra cepas de *Aspergillus* (*A. fumigatus*, *A. versicolor*, *A. ochraceus* y *A. niger*), *Trichoderma* (*T. víride*) y *Penicillium* (*P. funiculosum*, *P. ochrochloron* y *P. verrucosum* var. *cyclopium*). Entre las especies estudiadas

por sus propiedades antimicrobianas, algunas de ellas destacaron presentando resultados muy prometedores, como es el caso de *Suillus granulatus*, con valores de CMI, CMB y CMF inferiores a los antibióticos y anfifúngicos disponibles en el mercado.

El potencial antitumoral se evaluó frente a un amplio panel de líneas celulares tumorales humanas (MCF-7: adenocarcinoma de mama, NCI-H460: carcinoma de pulmón, HCT-15: adenocarcinoma colorrectal, HeLa: adenocarcinoma cervical, HepG2: carcinoma hepatocelular, y AGS: adenocarcinoma gástrico), realizando el ensayo de sulforodamina B (SRB) y calculando el porcentaje de inhibición del crecimiento celular (GI_{50}). Realizando el mismo ensayo colorimétrico (SRB), se evaluó también la citotoxicidad de los extractos en cultivos celulares primarios no tumorales obtenidos a partir de hígado porcino (PLP2), con el fin de obtener unos resultados preliminares respectante a su hepatotoxicidad. En un estudio más pormenorizado (artículo 9 de la presente tesis doctoral), se utilizó la línea celular humana no maligna de mama MCF-10A como control no tumoral. Todos los extractos estudiados revelaron efectos antiproliferativos en las líneas celulares ensayadas (con excepción de *Cordyceps militaris* frente a células HepG2) y no se observó citotoxicidad para las concentraciones ensayadas en las células no tumorales.

A partir de los resultados obtenidos en los ensayos anteriormente citados, se realizaron algunos estudios con el fin de establecer un posible mecanismo de acción antitumoral de los extractos de las setas estudiadas. Para estos ensayos se seleccionaron las especies *Leccinum vulpinum* y *Ganoderma lucidum*. En el caso de *L. vulpinum*, esta especie demostró diminuir la proliferación celular (ensayo de BrdU) e inducir la apoptosis (Western blot y citometría de flujo) en células de adenocarcinoma de mama (MCF-7). Además, los resultados también sugieren que el extracto de esta seta puede causa daños en el ADN celular (ensayo de Comet). Mientras que *G. lucidum* demostró inducir autofagia. Para ello, se transfectó una línea celular de adenocarcinoma gástrico (AGS) con un vector de expresión mCherry-LC3, y se sometió a tratamiento con el extracto metanólico de *G. lucidum*. Este tratamiento aumentó la formación de autofagosomas (vacuolas típicas de la autofagia), además de que los niveles celulares de LC3-II también aumentaron, y los niveles celulares de p62 disminuyeron (Western blot), lo que confirma que el extracto de *G. lucidum* afecta a la autofagia celular. Concretamente, podemos afirmar que el extracto de *G. lucidum* puede causar una inducción de la autofagia en lugar de una reducción en el flujo autofágico, ya que el tratamiento con el extracto junto con

inhibidores lisossómicos de proteasa (E-64d / pepstatin) provocó un aumento adicional de los niveles de LC3-II celular junto con un aumento en los niveles de p62 (Western blot).

Podemos concluir que en general, las setas de la región noreste de Portugal demostraron que son una matriz natural que se puede considerar como alimento y/o ingrediente funcional, ya que demostraron presentar diversios compuestos bioactivos que además de destacar por su funcion nutricional, presentan propiedades beneficiosas para la salud, destacando por su potencial antioxidante, antimicrobiano y antitumoral. Sin embargo, son necesarios estudios más profundos tales como la determinación del mecanismo de acción de estos compuestos tanto mediante técnicas *in vitro* como *in vivo*.

Dado que los datos obtenidos ponen de relieve el potencial de las setas como fuente de compuestos bioactivos, el presente estudio también contribuye a la concienciación de la conservación de los recursos micológicos no sólo por cuestiones ambientales, sino también por su potencial como fuente de compuestos nutracéuticos / farmacéuticos.

Part I

Introduction

1.1. Functional foods and nutraceuticals

Nowadays there are an increasing number of cases of several illnesses (*e.g.*, chronic diseases that can lead to cardiovascular problems), largely because the adopted lifestyle. Moreover, the rising cost of health care and pharmaceuticals, increased life expectancy and the consequent increase in the number of elderly people, as well as the demand for an improved quality of life, has led to an increased concern about food intake and an emergence of new concepts of nutrition (Ghosh et al., 2014). In fact, this is one of the concerns of the several official statements, such as the European Food Safety Authority (EFSA) and World Health Organization (WHO), whose commitment is to ensure that the foods consumed in Europe and worldwide are safe, and release information which help people to acquire healthy lifestyle habits associated with a balanced diet (EFSA, 2010; WHO, 2004).

The primary role of diet is to provide nutrients essential for metabolic needs but it also prevents malnutrition, promotes satiety and well-being and provides health benefits at physiological levels while ensuring optimal health and preventing disease (Alkerwi, 2014; Diplock et al., 1999). Thus, the quality of the diet is strongly related to the quality of life. Indeed, the diet plays a very important role in the diseases most prevalent in our society, including cardiovascular diseases (Estruch et al., 2013; McEvoy & Woodside, 2015), hypertension (Drenjančević-Perić et al., 2011; Sacks & Campos, 2010), or obesity (Ding et al., 2010; Hensrud, 2004) and cancer (Baena & Salinas, 2015; Gonzalez & Roboli, 2010).

Nowadays, it is well established that diets should be rich in fruits, vegetables, legumes, grains and nuts while the intake of free sugars and salt should be limited. By adopting this advice and avoiding sedentary behaviour, it is possible to attain energy balance and a healthy weight as well as to reduce the risk of some diseases (Ghosh et al., 2014; Gonzalez & Roboli, 2010; Key, 2011; Lock et al., 2010; Reis et al., 2017; WCRF/AICR, 2007; WHO, 2004; WHO/FAO, 2003).

Given the strong evidence linking diet quality to life quality and due to advances in food science and technology, new food concepts have been developed, including "functional foods" and "nutraceuticals". There is no consistent and universally accepted definition for functional foods. The difficulty in finding a definition (especially according to Europe and USA legislations) seems to be due to the fact that functional foods are considered as a concept

rather than a well-defined group of food products (Roberfroid, 2000; Alzamora et al., 2005; Siró et al., 2008; Ghosh et al., 2014). Consequently, there are many definitions for this term. The European Commission's Concerted Action on Functional Food Science in Europe (FUFOSE) defined functional food as "a food that beneficially affects one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. It is consumed as part of a normal food pattern. It is not a pill, a capsule or any form of dietary supplement" (EU, 2010).

Actually, in the broad sense, all foods can be functional, as they provide the nutrients and energy necessary for the body metabolism. Nonetheless, functional foods may regulate the energy balance and body weight, promote early development and growth, have cardiovascular and intestinal functions, defensive effects against oxidative stress, and/or enhance the physical and mental well-being, promoting long-term health (Diplock et al., 1999; Howlett, 2008). This way, a functional food can be (Diplock et al., 1999; Roberfroid, 2000; Howlett, 2008):

- A natural, unmodified food;
- A food in which one of the components has been enhanced through special growing conditions, breeding or biotechnological means;
- A food to which a component has been added to provide benefits;
- A food from which a component has been removed by technological or biotechnological means so that the food provides benefits not otherwise available;
- A food in which a component has been replaced by an alternative component with favourable properties;
- A food in which one or more components has been modified by enzymatic, chemical or technological means to provide a benefit;
- A food in which the bioavailability of one or more components has been modified;
- A combination of any of the above.

The use of food or food ingredients to promote health is well established (Ghosh et al., 2014; Howlett, 2008). However, it should be kept in mind that the disease state occurs due to a whole range of biological processes and, therefore, the functional food intake alone does not eliminate the risk of disease in general. So we cannot forget all the healthy habits (*e.g.*, avoid

a sedentary lifestyle, do not smoke, etc.), associating them with a healthy and balanced diet (Howlett, 2008).

Furthermore, functional foods and medicines/drugs should not be confused. If on one hand functional foods are ingested so as to restore, strengthen or maintain the normal physiology of the body, drugs are consumed to treat and/or prevent specific diseases, intervening in a disturbed physiological process. Unlike functional foods that are consumed as part of the normal diet, drugs are usually administered in precise doses, under medical supervision, as part of a controlled treatment, and often in the form of pills, tablets, capsules or syrups (Howlett, 2008).

Food supplements are also available, which are defined as foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities (Commition Directive 2002/46/EC). Once food supplements have a pharmaceutical form, and they are not consumed as traditional foods in daily diet, they cannot be regarded as functional foods. However, given that from a legal point of view, they are not considered as treating or preventing diseases, they still are governed by food laws (Howlett, 2008; Directive 2002/46/EC).

Finally, for food labelling proposes (commercial communications, including, *inter alia*, generic advertising of food and promotional campaigns) it should have scientifically based claims, which have been previously authorized by EFSA. It is necessary to ensure that the substances for which a claim is made have been shown to have a beneficial nutritional or physiological effect (*e.g.*, nutrient content claims, which refers to the levels of a nutrient contained in a food; it may be a source of certain minerals or high in fibre and unsaturated fatty acids) (Diplock et al., 1999; Roberfroid, 2000; Regulation (EC) No 1924/2006).

Unlike functional foods that, in most cases, their role is related with reducing the risk of diseases rather than preventing it, nutraceuticals appear as exerting a pharmacological role. So, although both have benefits for health, it is considered that the concept of functional foods belongs to nutrition and the concept of nutraceutical belongs to pharmacology (Ghosh et al.,

2014; Howlett, 2008; Kalra, 2003; Roberfroid, 2000). Nutraceuticals were first defined as "a food (or part of a food) that provides medical or health benefits, including the prevention and treatment of a disease" (DeFelice, 1993), and although there is no single definition for these products (as for functional foods), all of them are based on this statement. Once functional foods and nutraceuticals concepts are related, they are often confused and used incorrectly. Furthermore, according to some definitions, a functional food provides the essential and required nutrients to a healthy daily life. However, when a functional food aids in the prevention and treatment of certain diseases or sickness, shall be called nutraceutical (Kalra, 2003; Ghosh et al., 2014).

Consequently, nutraceuticals are usually consumed to promote well-being, through the prevention and/or treatment of diseases and/or disorders. They may be a food extract, a single natural compound or nutrient and not necessarily a complete food (*e.g.*, resveratrol, curcumin, vitamin E), which may be included in pharmaceutical form (pills, tablets, etc) as dietary supplements and as part of a specific diet (Gupta et al., 2010; Hardy, 2000; Kalra, 2003; Pandey et al., 2010; Reis et al., 2017; Sikora et al., 2010; Silk & Smoliga, 2014). Noted that, while exerting a pharmacological function, nutraceuticals, also known as pharmaconutrients, should not be confused with medicines, which are administered in precise doses, under medical supervision, to treat or prevent a specific disease (Hardy, 2000; Howlett, 2008). **Table 1** shows the main differences between functional foods, food supplements, nutraceuticals and medicines/drugs.

Table 1. General differences between functional foods, food supplements, nutraceuticals and medicines/drugs (Reis et al., 2017).

	Functional food	**Food supplement**	**Nutraceutical**	**Medicines**
Form	Food	Pill, tablet, capsule, syrup	Pill, tablet, capsule, syrup	Pill, tablet, capsule, syrup, injectables, etc.
Consumption	Consumed as part of the normal diet	Usually during a certain period of time	Daily consumed (usually for a period of time), once they are food constituents / extracts	Controlled doses according to medical prescription and for a predetermined period of time
Purpose	Exert a health or physiological effect; improvement of the state of health and well-being and/or reduction of the risk of disease	Typically consumed to ensure the intake of certain ingredient(s) (*e.g.*, vitamins, mineral, amino acids); They may also help to reduce the risk of disease	Promote well-being through the prevention and/or treatment of diseases and/or disorders	Pharmacological purpose; To treat a specific disease
Examples	- Natural food: mushrooms, nuts, tomatoes, fatty fish; - Processed food: dairy products and beverages (*e.g.*, yoghurts and energy-restoring drinks or teas) fortified with "functional ingredients" rich in vitamins, pro- and prebiotics, polyphenols, or plant sterol esters, such as margarines.	- Vitamin and mineral supplements	- Cod liver oil - Resveratrol - Reishi capsules	- *Amiloride* (diuretic) - *Omeprazole* (antiulcer)

According to their potential, nutraceuticals may be classified in two groups: potential nutraceuticals and established nutraceuticals (Pandey et al., 2010). The first group includes the products identified as potentially beneficial to health; from the moment in which there is scientific and clinical data showing their bioactivity, these become established nutraceuticals (Pandey et al., 2010). Unfortunately, most nutraceuticals still belongs to the first group, hence the need to perform more studies in this area. Surprisingly, the preference of these products over pharmaceuticals is well accepted by pharmaceutical and biotechnology companies (Kalra, 2003) and the interest in this field is increasing. This is due to the fast advances in scientific knowledge that attest to the vital role of diet in health and prevention of diseases, health costs have been rising dramatically over time, increased life expectancy, the advances in the food industry and the regulatory environment changing (Pandey et al., 2010).

However, this field still needs for more in-depth studies concerning the bioactivity and possible side effects of nutraceuticals. Furthermore, long-term clinical trials are also needed to scientifically validate nutraceuticals as biologically active. The interaction of nutraceuticals with food and drugs is also an area that should be further developed, as well as bio-availability tests and efficacy of nutraceuticals (Pandey et al., 2010).

Overall, an unbalanced diet can lead to the development of many diseases, as well as a proper diet can prevent many illness states. Based on this knowledge, emerge the concepts of functional foods and nutraceuticals, which aim, in addition to providing essential nutrients, is to promote healthy development and consumer welfare. Although often confused, the majority difference between these two concepts lies in that the functional food indeed play a nutritional role associated with preventive effects of diseases and sickness, while nutraceuticals can be used in an attempt to accomplish desired therapeutic outcomes with reduced side effects. It cannot be forgotten that a balanced diet alone is not enough for an optimal state of health. Healthy habits must be acquired and risky behaviours must be avoided (*e.g.*, sedentary lifestyle, smoking).

1.2. Mushrooms as functional foods and as a source for nutraceutical's development

When we think of a balanced diet and the healthiest means by which to achieve it, plants and plant products immediately come to mind, as well as mushrooms. Although their use has been reported for thousands of years, it has only been in recent years that the consumption of mushrooms has increased, mainly due to the increasing awareness that a stable and balanced diet exerts a key role in normal body functioning and sustaining health (Reis et al., 2017). Consumed for their texture, aroma and flavour, mushrooms are an excellent choice for both their nutritional value and medicinal properties (Valverde et al., 2015). Indeed, some authors consider mushrooms as "inherent functional foods" (Smith & Charter, 2011).

1.2.1. Bioactive compounds in mushrooms

As previously mentioned, in addition to their nutritional value, in recent years there was an increasing study of mushrooms as a source of bioactive compounds. Mushrooms have in their chemical constitution fatty acids (Gao et al., 2012), phenolic compounds, tocopherols, carotenoids, ascorbic acid, among others (Ferreira et al., 2009 and 2010; Lindequist et al., 2005; Roupas et al., 2012; Valverde et al., 2015) responsible for their bioactive properties.

Since mushrooms get their nutrients by absorption, they are toughly influenced by the environment in which they develop. This influence is reflected in their chemical composition which varies according to the region where they came from, as well as from species to species. However, some chemical properties are maintained within the genus/species. Indeed, there are some specific molecules of certain species, such as cordycepin (from the genus *Cordyceps*), ganoderic acid (from the genus *Ganoderma*) or agaritin (from the genus *Agaricus*). Thus, while the mushrooms are quite influenced by the environment, it seems that there is a chemical profile that is maintained and is typical of each species.

The following are some of the most bioactive compounds (hydrophilic and lipophilic) present and described in mushrooms.

Hydrophilic compounds

♦ Vitamin C – Ascorbic acid and dehydroascorbic acid

All vitamins are essential to our well-being (even in limited quantities), and as we are unable to synthesize them, we must obtain them through the diet.

Vitamin C is a water-soluble vitamin with a similar structure to glucose ($C_6H_8O_6$). In foods, vitamin C is mostly found as ascorbic acid, and then this is the nomenclature commonly used for this vitamin. However, we can also find vitamin C in its oxidized form, the dehydroascorbic acid (**Figure 1**) (Gropper & Smith, 2012; Hickey & Saul, 2008).

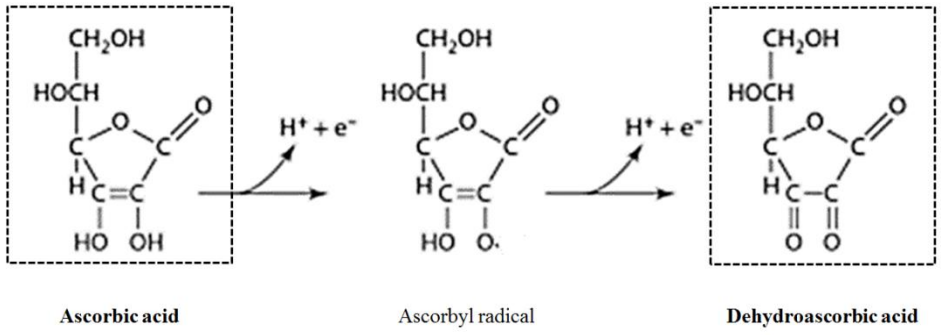


Figure 1. Chemical structure of the two forms of vitamin C (oxidation reaction; adapted from Gropper & Smith, 2012).

Ascorbic acid is absorbed throughout the small intestine assisted by the sodium-dependent vitamin C transporters SVCT 1 and SVCT 2. SVCT 1, the main responsible for vitamin C absorption, is down-regulated by ascorbic acid, which prevents the absorption of excessive and unnecessary amounts of this vitamin. Therefore, the absorption of vitamin C decreases with increased intake. Regarding the oxidized form of vitamin C, dehydroascorbic acid, it is absorbed by glucose transporters (mostly GLUT 1 and 3) in the gastrointestinal tract. When absorbed, it is reduced back to ascorbic acid (mediated through dehydroascorbic acid reductase) (Gropper & Smith, 2012).

Vitamin C performs important roles in human body, being well-known for its antioxidant properties, as an endogenous non-enzymatic antioxidant defence (Ferreira et al., 2009). In this context, it is effective in scavenging the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), singlet oxygen (O_2) and reactive nitrogen oxide ($^{\bullet}NO$). Vitamin C protects water soluble substances from oxidizing agents (DeBruyne et al., 2015). Moreover, it may regenerates already-oxidized substances to their original active form, such as vitamin E (Burke, 2007; Carocho & Ferreira, 2013; DeBruyne et al., 2015; Medeiros & Wildman, 2013). In addition, vitamin C protects iron from oxidation in the intestines, improving its absorption, and in cells and body fluids, it protects other molecules and even fat-soluble compounds, such as vitamins A and E and PUFA from oxidative processes (DeBruyne et al., 2015).

Vitamin C is also a cofactor in some important enzymatic reactions, playing central roles in many physiological processes in the human body, one of the most recognized, the collagen synthesis. Vitamin C is required for the posttranslational modifications of collagen, which allow aggregation and cross-linking of the chains (Gropper & Smith, 2012; Hickey & Saul, 2008; Medeiros & Wildman, 2013). Vitamin C is also required for carnitine synthesis, a molecule required for the transport of long-chain fatty acids into the mitochondria to produce energy (Gropper & Smith, 2012; Hickey & Saul, 2008), and is involved in the synthesis of amino acids such as tyrosine, and neurotransmitters such as serotonin (Gropper & Smith, 2012; Medeiros & Wildman, 2013).

Several illnesses increase vitamin C needs. For years that consumption of foods rich in vitamin C is recommended for colds/flu. Because of this, there are some suggestions for antihistamine activity by this vitamin. Moreover, the needs of this vitamin in stressful situations are also increased (DeBruyne et al., 2015).

Severe deficiency of vitamin C (total body vitamin pool falls below about 300 mg and plasma vitamin concentration drop to < 0.2 mg/dL) results in scurvy development. This disease has many symptoms; initially fatigue and malaise, which may be associated with impaired carnitine synthesis. Other symptoms are associated with the improper formation of collagen,

wound healing, inflamed and bleeding gums or capillary fragility (Fantuzzi, 2014; Gropper & Smith, 2012).

Besides its known antioxidant potential, there is also some evidence of the antiatherogenic and antitumour activities of vitamin C (Carr et al., 2000; Verrax & Calderon, 2009). Note that, although the intake of high concentrations of vitamin C (2000 mg / day or more) have been associated with antitumour effects, some studies have inferred a possible pro-oxidant and even an anti-carcinogenic effect at high concentrations (Li & Schellhorn, 2007; Naidu, 2003).

- **Organic acids**

Organic acids are weak acids, soluble in water and in organic solvents, comprising a group of substances having acidic properties. These acids are characterized by the carboxyl group (-COOH), which dissociates into a proton and the conjugated base, conferring them the acid properties. Since their main chemical feature is the presence of one or more carboxyl groups, they are also named carboxylic acids. Usually, they contribute to the organoleptic properties, stability and microbiological control in foods (Nollet, 2004; Nollet & Toldrá, 2015; Yildiz, 2009). They are classified depending on their chemical structure, namely the type of carbon chain, extent of saturation and substitution, and the number of carboxyl groups (**Figure 2**; Nollet, 2004; Yildiz, 2009).

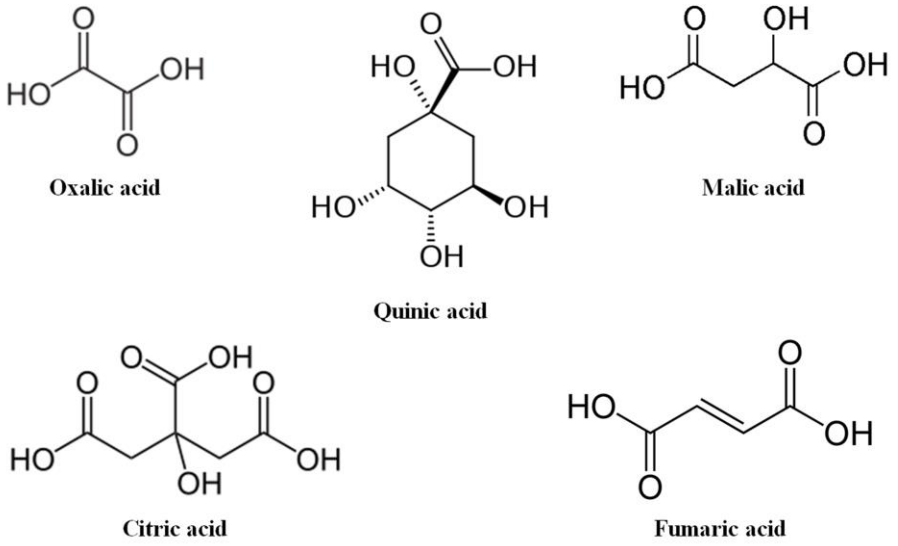


Figure 2. Chemical structure of some organic acids present in foods. Oxalic acid (2C), malic acid (4C), and fumaric acid (4C), dicarboxylic acids; quinic acid (7C), a monocarboxylic acid; and citric acid (6C), a tricarboxylic acid (based on Yildiz, 2009).

Organic acids may occur naturally in foods or may be incorporated to add or enhance some properties to food products. They are added as stabilisers, acidulants or preservatives, contributing for the sensorial properties of food products (Carocho et al., 2014; Nollet, 2004). For example, an additive widely used in the food industry, is citric acid (E-330), added to foods as a flavouring and preservative component (Lee, 2014).

Noted that some of the organic acids found naturally in mushrooms are intermediates in the Krebs cycle, also named citric acid cycle, one of the main metabolic pathways of primary metabolism in living beings (Hanson, 2008; Yildiz, 2009).

In **Table S1 (Annex 1)** it is possible to consult data referring to the studies that report the ascorbic acid contents and main organic acids found in Portuguese edible mushroom species. Ascorbic acid has not been reported in all the studied species. Regarding other organic acids, these are found in several species at varying concentrations. However, oxalic, malic and fumaric acids seem to be the most common acids present (the latter generally in lower amounts).

- **Phenolic acids and related compounds**

Phenolic compounds are part of the secondary metabolites produced by mushrooms. These compounds are characterized by one or more hydroxyl groups in their chemical constitution, attached directly to an aromatic ring (Vermerris & Nicholson, 2007), and are widely known for their bioactive potential.

Phenolic compounds range from relatively simple molecules, such as phenolic acids or flavonoids to large and complex ones, such as lignins and tannins. Indeed, there are several molecular groups of the phenolic compounds family, namely hydroxybenzoic acids, hydroxycinnamic acids, flavonoids or coumarins (Carocho & Ferreira, 2013; Vermerris & Nicholson, 2007). However, the present work will be focus in the first two classes, which are the most commonly found in mushrooms.

Hydroxybenzoic acids are produced through the shikimate pathway and are constituted by seven carbon molecules, with a C6-C1 skeleton, and characterized by the presence of a carboxyl group substituted on the phenol (**Figure 3**). This group includes gallic acid, *p*-hydroxybenzoic acid, and protocatechuic acid, among others (Vermerris & Nicholson, 2007). Whilst, hydroxycinnamic acids are derivatives of cinnamic acid, obtained through the phenilpropanoid pathway. These compounds are more common if foods comparing with hydroxybenzoic acids (Yildiz, 2009). Hydroxycinnamic acids present a C6-C3 skeleton with one or more hydroxyl groups, some of which may be methylated. Some of the most representative compounds of this group are *p*-coumaric, caffeic and ferulic acids (Vermerris & Nicholson, 2007).

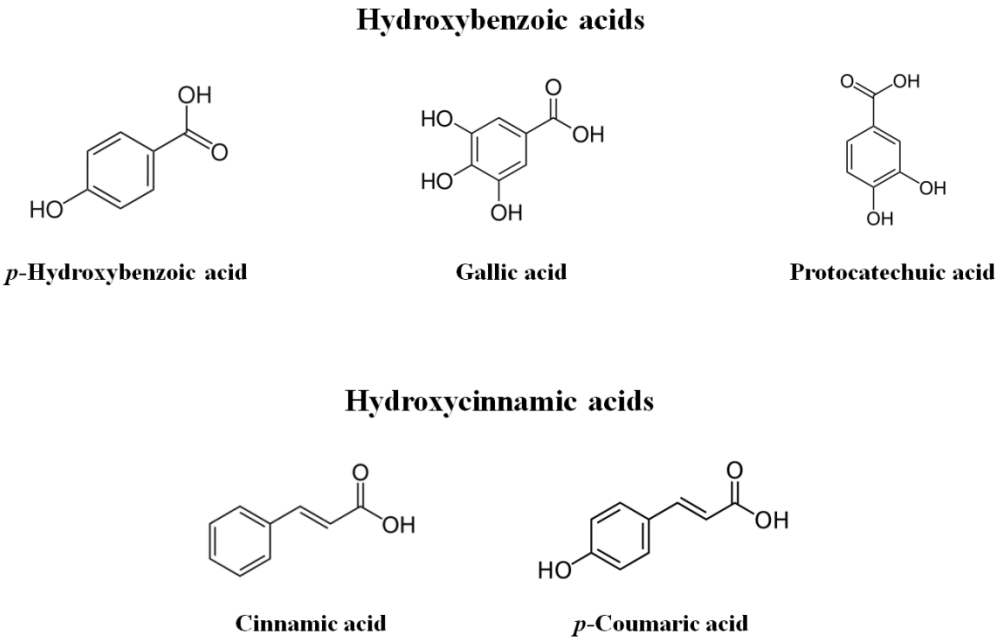


Figure 3. Chemical structure of some phenolic acids and related compounds.

Phenolic compounds are usually involved in the allelopathy mechanisms (Cheema et al., 2012). However, in addition of acting as signalling molecules against pathogens, they can also allow the establishment of symbiosis relationships (*i.e.*, mychorrizas) (Mandal et al., 2010; Reis et al., 2011a).

Phenolic acids are widely known for their antioxidant potential. Moreover, they have also been referred as having anti-inflammatory and anticancer properties. In human body free phenolic acids are mainly absorbed throughout the stomach and small intestine, and then metabolised in the liver. In this stage, they undergo methylation, glucuronidation and sulfation reactions, as well as are degraded into other acids or compounds (Ho et al., 2013). These alterations in their chemical structure may affect/alter their redox potential and/or their antioxidant activity. In some cases, the obtained metabolites are even more bioactive than the parental compounds. For this reason, it becomes important to perform some studies regarding the bioactive potential of these metabolites resulting from the body metabolism. The unabsorbed fraction is deesterified and dehydroxylated in the colon and further metabolized in the liver. The resulting metabolites are excreted through bile and urine (Ho et al., 2013).

Table S2 (Annex 1) shows the most common phenolic acids and related compounds found in Portuguese edible mushrooms. Generally, *p*-hydroxybenzoic acid is the main phenolic acid reported in the majority of the studied species, as well as the related compound cinnamic acid.

Overall, mushrooms have a great nutritional value, being a reasonably source of vitamins, such as vitamin C and E. Moreover, they have a great variety of bioactive compounds, such as phenolic acids. Therefore, mushrooms are an excellent source of many different nutraceuticals and might be definitely included in the human diet as a functional food product, as well as to promote health based on the synergistic effects of all their components.

Lipophilic compounds

- **Fatty acids**

Fatty acids consist in a hydrocarbon chain of variable length, with a carboxyl group (COOH) at one end (the delta (Δ) end) and a methyl group (CH_3) at the other end (the omega (ω) or *n*-end) (**Figure 4**). Besides varying in chain length, fatty acids can vary the degree of unsaturation, being classified as saturated (no double bonds), monounsaturated (single double bond) and polyunsaturated (several double bonds). Their nomenclature is also made according the position of the first double bond (*i.e.*, omega-3 and omega-6) and the configuration thereof (*cis* and *trans*) (Chow, 2007; Driskell, 2007; McGuire & Beerman, 2012; Sanders & Emery, 2003). Although the fatty acids mainly found in foods are composed of 14 to 22 carbon atoms, their backbone chain length can range 2 to 80 carbon atoms (Sanders & Emery, 2003).

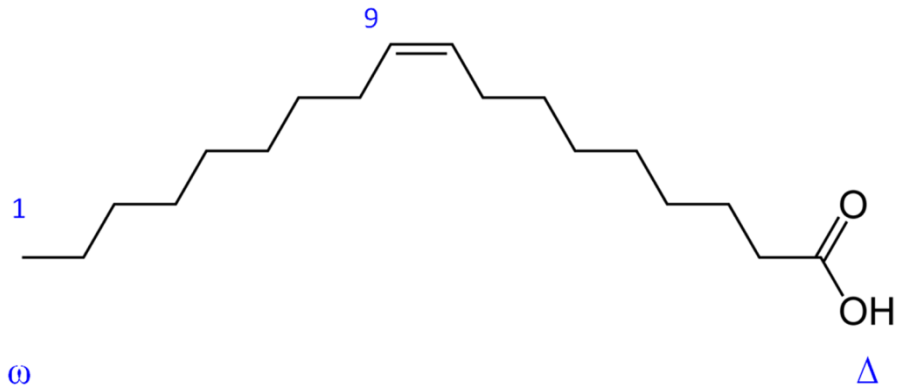


Figure 4. Chemical structure of oleic acid. It is possible to identify the delta (Δ) and omega (ω) ends; one double bond located at the *n*-9 position (the ninth carbon from the methyl end); and verify that the hydrogen atoms next to the double bond is on the same side of the carbon chain. Accordingly, oleic acid is a monounsaturated omega-9 fatty acid with a 18-carbon chain and one *cis*-double bond (Lipid number – C18:1*n*9).

Fatty acids, the most abundant lipids in the human body, result from the digestion of fats ingested through the diet. Fats are essentially digested in the small intestine, where they are reduced to fatty acids and glycerol, and subsequently absorbed. Fats metabolism occurs in cells and in the liver. In cells, the obtained energy is released through the breakdown of fatty acids. This metabolism leads to the formation of water and carbon dioxide that can be used by the cell or released by circulatory, respiratory and excretory systems. The fat that is not necessary to the cell at that time is stored as adipose tissue. In the liver triglycerides are hydrolysed to form new ones, depending on the metabolic needs (Roth, 2013).

Fatty acids, particularly the unsaturated fatty acids (mono and polyunsaturated), play many roles in the human body. Although their main functions are associated with the storage and energy supply, they also perform structural and metabolic functions especially at cellular membrane level. Fatty acids are stored in the form of triglycerides (three fatty acids chains attached to a molecule of glycerol) in adipose tissue, functioning as energy suppliers in case of need (*e.g.*, starvation conditions). In turn, the adipose tissue serves to support and protect the organs and bones, as well as protecting our body against low temperatures (Roth, 2013; The British Nutrition Foundation, 2013). Furthermore, fatty acids are an integral part the cells membranes, namely in the form of phospholipids, exerting structural functions and

contributing to the membrane flexibility, fluidity and permeability. Phospholipids can also circulate throughout the body, forming the lipoproteins complexes which help in digestion, absorption and transport of lipophilic compounds such as vitamin E, triglycerides or cholesterol (Kidd, 2007; Li et al., 2015; Roth, 2013). Fatty acids are also involved in metabolic processes underlying the response of cells to hormones, neurotransmitters and cell growth factors (The British Nutrition Foundation, 2013).

There are also some studies reporting fatty acids as having influence in reducing the risk of certain illnesses such as cardiovascular disease (Estruch et al., 2013; Gillingham et al., 2011), to have anti-inflammatory properties (Calder, 2015; Wall et al., 2010), even being associated to have beneficial effects in diseases such as cancer (Jing et al., 2013; Laviano et al., 2013).

There are two essential fatty acids, linoleic and α-linolenic acids. Linoleic acid is a polyunsaturated omega-6 fatty acid, while the latter is a polyunsaturated omega-3 fatty acid (**Figure 5**). In addition to being cellular membrane components, from these two essential nutrients, our organism synthesises new fatty acids, particularly all the other omega-6 and omega-3 fatty acids and other compounds, such as eicosanoids, with important functions in the body (*i.e.*, blood pressure, clot formation, blood lipid concentration, immune response, inflammatory response, among others). The longer-chain fatty acids derived from linoleic and α-linolenic acids (*e.g.*, arachidonic and docosahexaenoic acids) have also maintain the normal cellular function and regulation of gene expression, and some of their derivatives have also hormone-like effects, as is the case of eicosanoids that regulate the immune and cardiovascular systems and act as chemical messengers modulating several physiological functions (DeBruyne et al., 2015; McGuire & Beerman, 2012; Roth, 2013). Generally, omega-3 fatty acids have been particularly associated with a lower risk of heart disease, while omega-6 fatty acids have cholesterol-lowering effect (McGuire & Beerman, 2012; Roth & Townsend, 2003).

Since the essential fatty acids are stored in adipose tissue, their deficiency is rare. However, this deficiency may arise in persons with fragile health and, in this case there may be infections and slow wound healing processes (McGuire & Beerman, 2012).

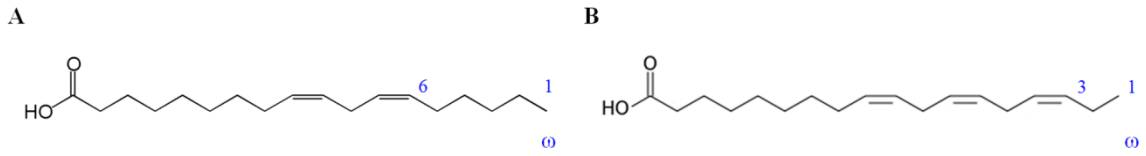


Figure 5. Chemical structure of the essential fatty acids. A- Linoleic acid (LA), a polyunsaturated omega-6 fatty acid with a 18-carbon chain and two *cis*-double bonds (Lipid number – C18:2*n*6); B- α-Linolenic acid (ALA), a polyunsaturated omega-3 fatty acid with a 18-carbon chain and three *cis*-double bonds (Lipid number – C18:3*n*3).

Although the fat content in mushrooms is low, it is possible to find a wide range of fatty acids in mushrooms. Though, some are dominant, particularly the saturated palmitic acid (C16:0), the unsaturated oleic acid (C18:1), and predominantly the polyunsaturated linoleic acid (LA, C18:2*n*6) (Cheung, 2008; Guillamón et al., 2010; Kalač, 2009 and 2013). Although the values can undergo some fluctuations according to the species under study, generally unsaturated fatty acids predominated over saturated fatty acids, which make mushrooms interesting food choices. Some data state that at least 72% of total fatty acids have been found to be unsaturated (Miles & Chang, 2004). More recent studies report that 50 – 78% of the total percentage of fatty acids comprises linoleic acid; 6 – 20% oleic acid and 11 – 26% palmitic acid (OECD, 2015). According to the WHO, there is convincing evidence that palmitic acid contributes to an increases risk of developing cardiovascular disease (WHO/FAO, 2003). On the contrary, French et al. demonstrated in 2002 that palmitic acid has no hypercholesteraemic effect if the intake of LA is greater than 4.5% of total energy. However, although this is one of the majority fatty acids found in mushrooms, its amount is far below compared to oleic acid (an omega-9 fatty acid) or linoleic acid (an omega-6 fatty acid). These fatty acids, as unsaturated fatty acids in general, are often reported as being potentially beneficial to health, being associated with a low risk of inflammatory processes, cardiovascular disease or cancer development (Baum et al., 2012; Cardoso et al., 2011; Carrillo et al., 2012; Czernichow et al., 2010; Sales-Campos et al., 2013). It is recommended to consume a minimum of 1% energy as n-6 PUFA and 0.2% energy as n-3 polyunsaturated fatty acids. Adequate intakes for male adults must be around 14-17 g per day of LA and 1.6 g per day of ALA, whereas for females the adequate intakes are lower, around 11-12 g per day of LA and 1.1 g per day of ALA

(Trumbo et al., 2002). Hence, mushrooms have a variety of fatty acids, mainly MUFA and PUFA, being a source of essential fatty acids (**Table 2**).

Table 2. Some of the fatty acids commonly found in mushrooms (Reis et al., 2014a)

Fatty acid	**Lipid number (C:D form)**
Caproic acid	C6:0
Caprylic acid	C8:0
Capric acid	C10:0
Lauric acid	C12:0
Tridecylic acid	C13:0
Myristic acid	C14:0
Myristoleic acid	C14:1
Pentadecanoic	C15:0
Palmitic acid	**C16:0**
Palmitoleic acid	C16:1
Heptadecanoic acid	C17:0
Stearic acid	C18:0
Oleic acid	**C18:1*n*9**
Linoleic acid	**C18:2*n*6**
α-Linolenic acid	C18:3*n*3
Arachidic acid	C20:0
Eicosenoic acid	C20:1
Eicosadienoic acid	C20:2
Eicosatrienoic acid	C20:3*n*3
Heneicosanoic acid	C21:0
Eicosapentaenoic acid	C20:5*n*3
Behenic acid	C22:0
Erucic acid	C22:1*n*9
Tricosanoic acid	C23:0
Lignoceric acid	C24:0
Nervonic acid	C24:1

♦ Vitamin E – Tocopherols and tocotrienols

Vitamin E is the term used for a family of chemically related compounds, tocopherols and tocotrienols, which share a common structure consisting of chromanol head/ring and a prenyl side chain. Vitamin E is therefore constituted by eight chemical compounds: four tocopherols (α-, β-, γ- and δ-isoforms) and four tocotrienols (also with α-, β-, γ- and δ-isoforms) (Kamal-Eldin & Appelqvist, 1996; Munné-Bosch & Alegre, 2002). These lipid-soluble molecules differ between them only in the degree of saturation of their hydrophobic prenyl side chains (**Figure 6**).

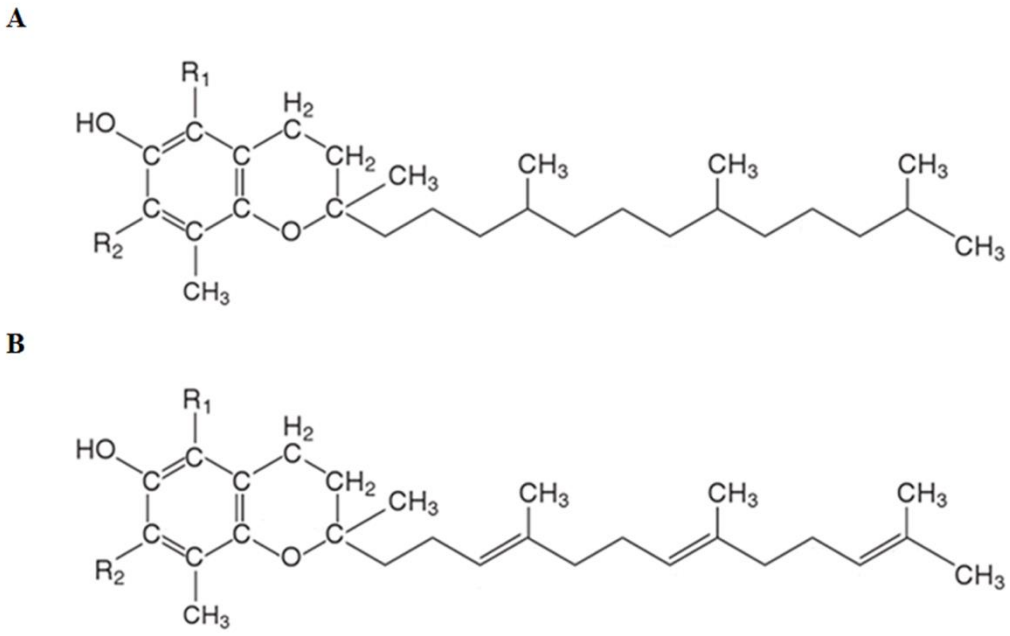


Figure 6. General chemical structure of vitamin E isoforms. A- Tocopherols (α-tocopherol: R^1 and R^2 = Me; β-tocopherol: R^1 = Me and R^2 = H; γ-tocopherol: R^1 = H and R^2 = Me; δ-tocopherol: R^1 and R^2 = H). B - Tocotrienols (α-tocotrienol: R^1 and R^2 = Me; β- tocotrienol: R^1 = Me and R^2 = H; γ-tocotrienol: R^1 = H and R^2 = Me; δ- tocotrienol: R^1 and R^2 = H) (Modified from Stipanuk & Caudill, 2013).

Tocopherols have been recognized as one of the most important antioxidants. This is due to their ability to protect the cell membranes, inhibiting the production of lipid peroxyl radicals induced by reactive oxygen species (ROS), thereby protecting cells from peroxidation of polyunsaturated fatty acids (PUFA) in the phospholipid membrane from oxidative damage (Fang et al., 2002; Gropper & Smith, 2012; McGuire & Beerman, 2012). The mechanism of action of vitamin E as antioxidant is well known. During the process of lipid peroxidation, PUFA (LH) reacts with free hydroxyl radicals (˙OH) or with molecular oxygen (O_2), leading to the formation of alkyl radicals (L˙) and water or alkyl radicals and the hydroperoxyl radical (HO_2˙), respectively (**Equation 1**).

$$LH + {}^{\cdot}OH \rightarrow L^{\cdot} + H_2O;$$
$$LH + O_2 \rightarrow L^{\cdot} + HO_2{}^{\cdot}$$

Equation 1. Two pair reactions involved in lipid peroxidation, depending on the reaction of PUFA with hydroxyl radicals or with molecular oxygen.

In turn, alkyl radicals may react with molecular oxygen in a propagation reaction to form a lipid peroxyl radical (LOO˙), which promotes lipid peroxidation (**Equation 2**).

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$$

Equation 2. Reaction of alkyl radicals with molecular oxygen.

This radical reacts with other organic compounds, such as other PUFA located in the cell membrane, arresting a hydrogen atom from these and generating stable lipid peroxides (LOOH) (Gropper & Smith, 2012). However, tocopherols located in or near the membrane surface may react with these radicals. Thus, tocopherols (TOH) react with lipid peroxyl radicals (LOO˙) and generate more stable products (**Equation 3**).

$$LOO^{\cdot} + TOH \rightarrow LOOH + TO^{\cdot}$$

Equation 3. Reaction of lipid peroxyl radical with other compounds producing stable products.

Tocopherols may also react directly with alkyl radicals ($L^{\cdot}$ + TOH → LH + $TO^{\cdot}$) or with alkoxy radicals ($LO^{\cdot}$), which are formed during the propagation process ($LO^{\cdot}$ + TOH → LOH + $TO^{\cdot}$) (Gropper & Smith, 2012; Kamal-Eldin & Appelqvist, 1996; Niki, 2014). After these reactions, tocopherols become oxidized, and the resulting radical (tocopheroxyl radical; $TO^{\cdot}$) reacts with other peroxyl radicals to form inactive compounds, such as tocopherylquinone (Gropper & Smith, 2012). The tocopheroxyl radical may also be reduced, which will allow the reuse of vitamin E. As abovementioned, this vitamin is regenerated by vitamin C, but also by other molecules such as GSH (reduced glutathione), or NADPH (Gropper & Smith, 2012).
Since vitamin E is involved in oxidative stress, protecting cells from free radical-induced oxidative damage, some authors infer that it can function as prevention of many diseases, such as heart disease or even cancer (Gropper & Smith, 2012; Ju et al., 2010). α-Tocopherol has long been considered the most active form of vitamin E (Kamal-Eldin & Appelqvist, 1996). However, other isoforms have been also recognized as bioactive. For example, γ-tocopherol is more effective suppressing reactive nitrogen species (RNS), has higher anti-inflammatory potential, and has been pointed as being more effective preventing cancer-related processes (Brigelius-Flohé, 2006; Hensley et al., 2004; Yang et al., 2012a). Although there are fewer studies on the specific activities of tocotrienols, these vitamers have been mentioned as having positive effects particularly in neurodegeneration process (Brigelius-Flohé, 2006), as well as anticancer and cholesterol-lowering properties (Colombo, 2010). Moreover, some studies reported that the corresponding tocopherols and tocotrienols isoforms exert the same antioxidant activity against lipid peroxidation in solution and liposomal membranes (Yoshida et al., 2003), and others suggests that, in fact, their antioxidant activity is higher than that of tocopherols (Packer et al., 2001). This could be due to the fact that tocotrienols are more readily transferred and incorporated into the cell membranes than tocopherols. However, though its bioavailability still remains poorly understood, it is known that tocotrienols plasma concentrations are much lower compared to tocopherols (Fu et al., 2014; Yoshida et al., 2003).

Vitamin E is hydrolysed and absorbed in the intestine, and as a fat-soluble vitamin, this process depends of the normal function of pancreas, since it requires biliary and pancreatic secretions in order to form micelles that will allow vitamin uptake by the intestine. After this course, vitamin E becomes solubilised, and the resulting micelles are then wrapped by

enterocytes through passive diffusion and incorporated together with other lipids, in chylomicrons which are secreted into the lymphatic system (Eggermont, 2006; Litwack, 2007; Rigotti, 2007; Stipanuk & Caudill, 2013; Traber, 2007). This seems to be the main via through which vitamin E is absorbed in the intestine. Lastly, vitamin E is secreted into the plasma (Litwack, 2007; Rigotti, 2007; Stipanuk & Caudill, 2013). Once incorporated in chylomicrons, vitamin E and other lipids will be transported to adipose tissue and muscles where takes place a partially digestion (lipolysis) of chylomicrons, that results in "remnant chylomicrons" free of triglycerides and enriched in cholesterol and with the four isoforms of tocopherols. The remnant chylomicrons are then taken up by hepatocytes (after binding to specialised low-density lipoprotein (LDL)-related receptors), where α-tocopherol is preferentially retained by the action of α-tocopherol transfer protein (α-TTP). This protein regulates the levels of vitamin E in the blood. Therefore, it is also responsible for the secretion of α-tocopherol from the liver to the plasma. α-TTP has higher affinity with α-tocopherol; therefore, this is the isoform preferentially transferred to the plasma, which explains the higher concentrations of this α-tocopherol in human body, comparing with the other isoforms (Eggermont, 2006; Rigotti, 2007; Traber, 2007). In the circulation, α-tocopherol is assembled into the very low-density lipoprotein (VLDL) and LDL particles and released for use by the peripheral tissues (Eggermont, 2006; Rigotti, 2007).

Vitamin E is not accumulated. It is metabolized similarly to xenobiotics, being excreted in urine or bile. It is estimated that about half of the tocopherols consumed throughout the diet is absorbed, being the remainder excreted in the faeces (Eggermont, 2006; Stipanuk & Caudill, 2013; Traber, 2007).

The deficiency in this vitamin is rare in people. However, it might occur in premature babies or people with fat malabsorption problems. Vitamin E, namely α-tocopherol deficiency, may cause peripheral neuropathy or blood disease problems (*i.e.*, increased erythrocyte hemolysis) (Roth, 2013; Traber, 2007).

Overall, vitamin E is an important exogenous lipophilic radical-scavenging antioxidant, preventing the auto-oxidation of PUFAs in cell membranes. Therefore, the intake of vitamin E depends on the intake of diets rich in PUFA; higher amounts of vitamin E are required when PUFA intake is high (Alfin-Slater & Kritchevsky, 2012; Bijlani & Manjunatha, 2010). Vitamin E antioxidant potential is well known and, probably due to its effectiveness as antioxidant, holds other properties, namely anti-inflammatory potential (Tahan et al., 2011).

Vitamin E, as vitamin C, is sensitive to different abiotic factors being easily degraded by light, water and heat (Wang et al., 1993).

1.2.2. Bioactivity of mushrooms

1.2.2.1. Antioxidant activity

In the human body (as in other aerobic organisms) free radicals and other reactive species are continually being produced during the normal cellular metabolism. This may occur by "accident" (*e.g.*, the leakage of electrons from the mitochondrial electron transport chain) or with some required purposes, such as involvement in cell signalling and homestasis processes or defence against pathogens (Ferreira et al., 2009; Halliwell, 2011; Halliwell & Gutteridge, 2015). These reactive species are very unstable, so the exposure of the body to these species leads to the development of endogenous defence mechanisms (enzymes and non-enzymatic molecules) in order to eliminate them (Ferreira et al., 2009; Goetz & Luch 2008; Valko et al., 2007). Therefore, in normal physiological conditions, the production of reactive species is maintained in balance by these antioxidant defence mechanisms. However, this balance may be affect by several factors (*e.g.*, decrease in antioxidants production or exposure to high levels of oxygen, UV and pollution exposure), and when this unbalanced situation tends to an increased production of free radicals (over the capability of the cell to orchestrate an effective antioxidant response), the body comes under oxidative stress (Ferreira et al., 2009; Halliwell & Gutteridge, 2015; Silva & Coutinho, 2010).

A free radical is defined as any atom or molecule that has unpaired electrons in the outer orbit (Gutteridge & Halliwell, 2000; Halliwell & Gutteridge, 2015). Therefore, these radicals are very unstable, and tend to capture an electron from stable neighboring molecules, so that they become electrochemically stable. Free radicals may derive from three elements, namely oxygen, nitrogen and sulphur, producing ROS, RNS and RSS, respectively. ROS are the most important class of reactive species originated in biological systems. These include the superoxide anion ($O_2^{\bullet -}$), which is considered a "primary ROS", the hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2), defined as "secondary ROS" (Carocho & Ferreira, 2013; Ferreira et al., 2009).

The oxidation mechanism is divided into three stages: initiation, propagation and transformation. In the first stage, free radicals are formed, which leads to an overall increase in the number of these species (**Equation 4**).

$$X^{\cdot} + LH \rightarrow L^{\cdot} + XH$$

Equation 4. Initiation stage of the oxidation mechanism.

In the propagation stage, the number of free radicals is maintained, once the free radicals formed in the first stage react with other molecules, and the result is one new free radical (**Equation 5**).

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$$
$$LOO^{\cdot} + LH \rightarrow L^{\cdot} + LOOH$$

Equation 5. Propagation stage of the oxidation mechanism.

Finally, the termination reactions (**Equation 6**) are those resulting in a decreased number of free radicals, since two free radicals combine to form a more stable molecule (Divya & Pandey, 2014; Shils et al., 2006).

$$LO^{\cdot} + LO^{\cdot} \rightarrow \text{non-radical polymers}$$
$$LOO^{\cdot} + LOO^{\cdot} \rightarrow \text{non-radical polymer}$$
$$L^{\cdot} + L^{\cdot} \rightarrow \text{non-radical polymers}$$
$$LOO^{\cdot} + L^{\cdot} \rightarrow \text{non-radical poltmers}$$

Equation 6. Transformation stage of the oxidation mechanism.

As abovementioned, in normal physiological conditions there is a balance between free radicals production and antioxidant defences. These antioxidant defences can be endogenous or acquired through the diet. Regarding the endogenous defences, these may be enzymatic such as superoxide dismutase, catalase or glutathione peroxidase. Cells also have non-enzymatic antioxidants, such as glutathione, minerals (such as zinc and selenium), or the

coenzyme Q10. It is also possible to obtain antioxidants through the diet, such as carotenoids, vitamins and phenolic acids (Carocho & Ferreira, 2013).

Antioxidants play their role through different mechanisms: i) inhibiting free radical oxidation reactions, through inhibiting the formation of free lipid radicals; ii) interfering with the propagation of the autoxidation chain reaction; iii) singlet oxygen quenchers; iv) acting synergistically with other antioxidants; v) converting hydroperoxides or pro-oxidants into stable compounds; vi) inhibitors of pro-oxidant enzymes (Carocho & Ferreira, 2013).

As shown in **Figure 7**, free radicals have different causes and different cellular targets, namely proteins, carbohydrates, lipids and nucleic acids. For this reason, many disease conditions have been associated with oxidative stress, such as atherosclerosis (Fearon & Faux, 2009), diabetes (Jaganjac et al., 2013), and cancer (Vera-Ramirez et al., 2011).

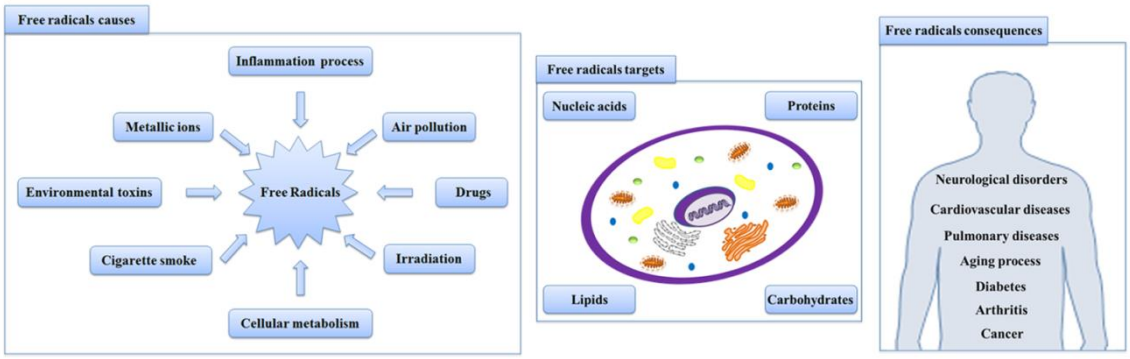


Figure 7. Main causes for the overproduction of free radicals, probable cellular targets and main consequences in human health associated to oxidative stress (Reis et al., 2017).

For this reason, there has been an increasing concern with regard to obtaining antioxidant compounds. Natural compounds have emerged as key candidates due to recognition of the inverse relationship between dietary intake of natural antioxidants and the incidence of human diseases. This has lead to an increased worldwide interest in using natural antioxidants in food, cosmetic, and pharmaceutical industries (Lobo et al., 2010; Taofiq et al., 2016; Valverde et al., 2015).

Mushrooms have been reported as a source of several antioxidants (Asatiani et al., 2010; Ferreira et al., 2009; Witkowska et al., 2011). **Table S3 (Annex 1)** lists the edible mushroom

species from the Portuguese mycoflora that have been identified as having antioxidant properties.

1.2.2.2. Antimicrobial activity

The term antimicrobial comprises a wide variety of pharmaceutical agents, which include antibacterial, antifungal, antiviral and anti-parasitic drugs (Leekha et al., 2011). The treatment of bacterial infections is extremely important. Despite the wide variety of existing antibiotics (*e.g.*, aminoglycosides, β-lactams, oxazolidinones, lincosamides, quinolones), treating bacterial infections has become an increasingly complicated task due to the development of bacterial resistance in recent years due to the inappropriate use of antibiotic drugs (Gualerzi, et al., 2013). Antibiotic drugs typically act by interfering with the synthesis of the bacterial cell wall, inhibiting protein or nucleic acid synthesis, inhibiting metabolism or breaking the bacterial membrane (Gallo et al., 2013; **Figure 8**).

Bacterial resistance can be intrinsic or innate, which means that the bacteria species are resistant to certain classes of antimicrobial agents. Moreover, the resistance may be acquired, which means that populations that were initially sensitive become resistant to an antibacterial agent and were able to proliferate in its presence. This resistance state may be developed by the acquisition of genes encoding enzymes that destroy the antibacterial agent before it can exert its effect; bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target; or bacteria can acquire different genes to perform an alternative metabolic pathway, which will result in the synthesis of modified bacterial cell walls that does not contain the binding site of the agent; besides, bacteria can also suffer mutations which restrict the access of the antimicrobial agents to the intracellular targets (Tenover, 2006). For all these reasons, the development of antibiotics has been one of the most important scientific challenges of the last years.

Because of the resistance phenomena, bacterial diseases that were previously treated may constitute serious problems today. For example, some of the microorganisms that have developed resistance to antibiotics are *Klebsiella* spp. and *Escherichia coli*. These bacteria species are the most important causal agents of Gram-negative bacteremia (presence of bacteria in the blood) both in hospitals and the community (Peralta et al., 2012). These microorganisms produce extended spectrum beta-lactamases (ESBL) that cause resistance to

most of the beta-lactam antibiotics, such as penicillins, monobactams and most cephalosporins. To treat such, known or suspected to be caused by multidrug-resistant bacteria, the use of wide spectrum antibiotics, fundamentally carbapenems, is recommended (Alves et al., 2012; Peralta et al., 2012). Other well-known Gram-negative group of bacteria that developed multi-antibiotic resistance is *Pseudomonas* spp. Although this genus is a common inhabitant of aquatic environments including drinking water, *Pseudomonas* spp. includes species considered opportunistic pathogens that can colonize animals and humans. *Pseudomonas aeruginosa* is the most studied pathogen from this genus and it can cause severe infections either in healthy or in immunocompromised persons (Mena & Gerba, 2009). Other species such as *Pseudomonas fluorescens*, *Pseudomonas stutzeri* or *Pseudomonas putida* may also be associated with human infections (Ana et al., 2010; Vaz-Moreira et al., 2012; Yoshino et al., 2011). This genus is resistant to aminoglycosides, carbapenemics and/or cephalosporins (Alves et al., 2012). Regarding Gram-positive bacteria, MRSA (Methicillin-resistant *Staphylococcus aureus*) is widely known as a common cause of bloodstream and other infections in hospital patients along with vancomycin-resistant *Enterococci* (Alves et al., 2012; Rice, 2006; Segal-Maurer et al., 1996).

Pathogenic fungi also cause injury in humans, animals, crops, and other living organisms (Alves et al., 2013). Although fungal infections contribute substantively to human morbidity and mortality, and despite the need for efficient diagnostic tests and new medicines and vaccines, research on the pathophysiology of human fungal infectionsthat cause disease falls short of other caused by other pathogens (such as the aforementioned bacteria) (Brown et al., 2012). The main pathogens responsible for fungal infections are dermatophytes, yeasts and moulds, being the most illnesses caused by pathogenic fungi, the superficial infections of the skin and nails. Actually, skin mycosis affects approximately 25% of the world's population (Havlickova et al., 2008). Dermatophytes belong essentially to three genera: *Trichophyton*, *Microsporum* and *Epidermophyton*. The genus *Trichophyton* is known to cause athlete’s foot (tinea pedis); *Microsporum canis* is one of the best known species from the *Microsporum* genus, which in addition to causing ringworm in pets, is also a widespread agent of tinea capitis in humans (ringworm of the hair/scalp); *Epidermophyton* species such as *Epidermophyton floccosum* are also known to cause tinea, namely tinea pedis and tinea cruris (ringworm of the groin) (Havlickova et al., 2008).

Among the yeast-like fungi *Candida* spp. is one of the most common infectious agents. Furthermore, the last data available (2011-2012), indicate this genus as the 5th most common pathogen associated with bloodstream infections (BSIs), isolated in 7.4% of all documented cases (European Centre for Disease Prevention and Control, 2016). Although *Candida* species are part of the transient or commensal flora in specific regions of the body, they become pathogenic to humans under particular systemic and local conditions (*e.g.*, when mucosal barriers are disrupted or the immune system is compromised). These species, including the well-known *Candida albicans*, can cause infection in skin, nails, mucous membranes and gastrointestinal tract (Havlickova et al., 2008), besides the BSIs in hospitalized patients. This infection (candidemia) has increased over the past few decades and is a challenge for the clinic. This is because it is caused by *Candida* species other than *Candida albicans*, namely *Candida glabrata* and *Candida krusei*, which tend to be more resistant to fluconazole, that is a reliable, cost-effective, and clinically safe antifungal agent against *Candida* infections (Kourkoumpetis et al., 2011; Martins et al., 2015; Pfaller et al., 2011; Puig-Asensio et al., 2014).

Fungal contamination is also closely associated with agricultural products. The products spoilage by fungi may result in the decrease of quality and yield of crops (*e.g.*, reduced palatability and the loss of nutritive value), as well as huge economic losses. After fungal attack, the crops may be contaminated with toxic secondary metabolites produced by fungi, known as mycotoxins. This contamination of food and feed crops may occur in various stages of the food chain (Bryden, 2012; Zain, 2011). Contamination of standing crops and stored feeds with mycotoxins is dependent on the fungi present, the agronomic practices, the composition of the commodity and the conditions of harvesting, handling and storage (Bryden, 2012). Among the best known and widely studied moulds that mostly contaminate agricultural products and produce mycotoxins, are the *Penicillium*, *Aspergillus* and *Fusarium* genera (Commission Regulation (EC) No 401/2006). Besides their metabolic versatility with great biotechnological potential, having many uses for the production of several food and non-food by-products (*e.g.*, production of the antibiotic penicillin by *Penicillium* species, large-scale obtaining of citric acid carried out by *Aspergillus niger* or *Fusarium venenatum* production to use as a meat substitute), these genera also include the foremost producers of mycotoxins (*e.g.*, *Aspergillus flavus* and *Aspergillus parasiticus* producers of aflatoxins; or

Aspergillus ochraceus, *Aspergillus carbonarius*, and *Penicillium verrucosum* producers of ochratoxin A) (Reis et al., 2017).

Exposure to mycotoxins is mostly by ingestion of contaminated foods, as well as by dermal contact or inhalation of toxic spores. The diseases caused by the exposure to mycotoxins are known as mycotoxicoses. As all toxicological syndromes, mycotoxicoses can be categorized as acute or chronic (Zain, 2011). Acute toxicity generally has a quick onset and an evident toxic response (*e.g.*, hemorrhagic necrosis of the liver or kidney disorders), while chronic toxicity is characterized by low-dose exposure over a long period of time, resulting in commonly irreversible effects (*e.g.*, hepatocellular carcinoma) (Zain, 2011).

The economic impact of mycotoxins include loss of human and animal life, increased health care and veterinary care costs, reduced livestock production, discarding contaminated foods and feeds, and investment in research and applications to reduce severity of the mycotoxin problems (Zain, 2011). It should be noted that guidelines exist to monitor/control the levels of mycotoxins in foodstuffs (Commission Regulation (EC) No 401/2006), to ensure the safety of consumption.

Figure 8 shows the main antibacterial and antifungal agents and their mechanism of action, namely their target on the microorganism's cells.

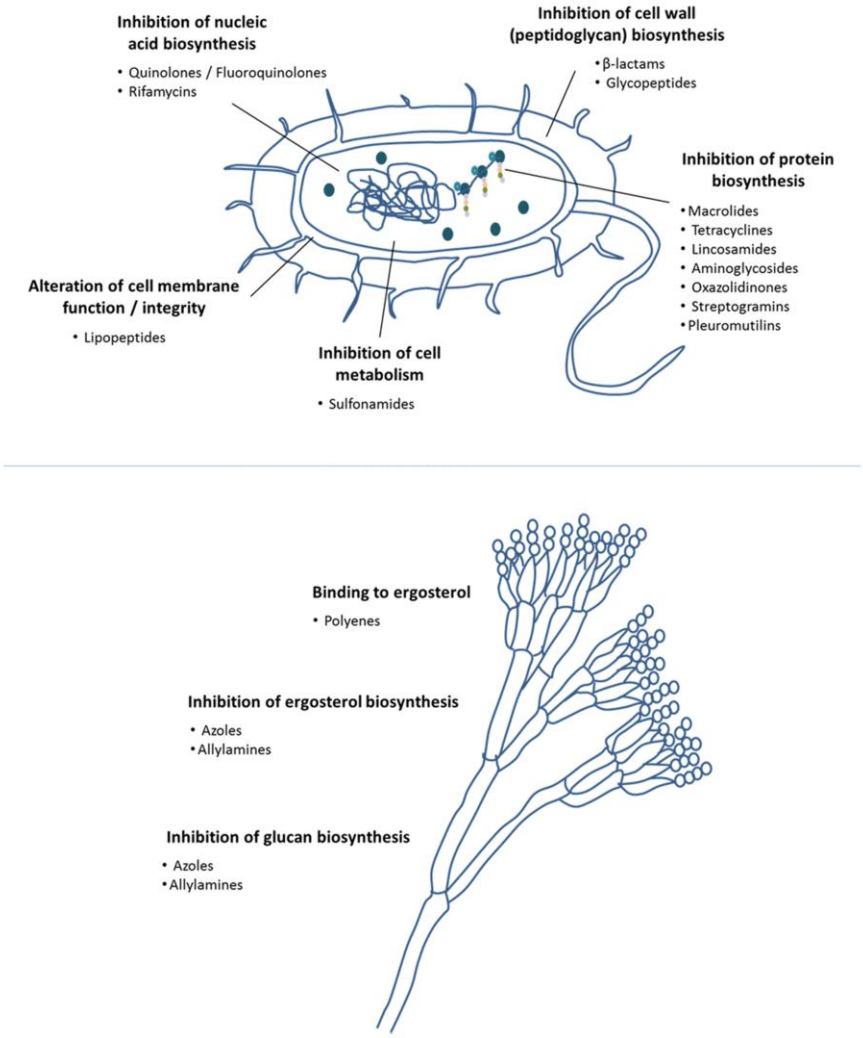


Figure 8. Mechanism of action of the antibacterial and antifungal agents used in clinical use. Antibacterial agents exert their effect mainly by 5 ways. They inhibit the cell wall synthesis interfering with peptidoglycan (cell wall component) synthesis, which will affect the rigidity and integrity of the cell wall leading to cell lysis / death. Antimicrobials may also alter the function or integrity of the cell membrane, disorganizing its permeability so that nucleic acids and cations leak out and the cell dies. Regarding the interaction with nucleic acids, antimicrobials may inhibit nucleotide synthesis or their interconversion, prevent DNA from functioning as an appropriate template; and they may also interfere with the polymerases involved in the DNA replication and transcription. Antimicrobials also inhibit protein synthesis interacting directly with the ribosome, binding to one or both 50S and 30S subunits. The metabolism of the cell may also be affected by antimicrobials, such as sulfonamides that interfere with folate metabolism. This class of antimicrobials, competitively blocking the biosynthesis of tetrahydrofolate, which is necessary for the ultimate synthesis of DNA, RNA and bacterial cell wall proteins. Regarding the antifungal agents, they can form a complex with ergosterol compromising the cell membrane fluidity. Other antifungals, such as azoles, inhibit the synthesis of this sterol. They are 14α-demethylase inhibitors, an enzyme that catalyzes a critical reaction of the sterol biosynthetic pathway, leading to the depletion of ergosterol in the membrane. Finally, other antifungal classes inhibit glucan biosynthesis, acting as specific non-competitive inhibitors of β-(1,3)-glucan synthetase (Reis et al., 2017).

Some studies suggest mushrooms as inhibitors of mycotoxins production. Culture filtrates from *Lentinula edodes* (Berk.) Pegler and *Trametes versicolor* (L.) Lloyd have revealed promising aflatoxin-inhibiting effects (Reverberi et al., 2005; Zjalic et al., 2006).

Some mushroom extracts, particularly obtained from *L. edodes* and *Phellinus linteus* (Berkeley & Curtis) Teng, have been reported as effective even against highly resistant bacteria, such as MRSA (Hearst et al., 2009; Hur et al., 2004). Besides, both primary and secondary metabolites from mushrooms have been associated with their antimicrobial properties, such as oxalic acids, peptides or proteins, and terpenes, steroids or benzoic acids derivatives (Alves et al., 2012).

Table S4 (Annex 1) lists the Portuguese mushroom species already described for its antibacterial potential, while **Table S5 (Annex 1)** lists the Portuguese mushroom species already described for its antifungal potential.

1.2.2.3. Antitumour activity

Cancer is the leading cause of death in developed countries. The most frequently occurring forms of the disease in the European Union are breast, prostate, lung and colorectal cancers. Lung cancer is the most frequent cause of cancer death in men, while in women, is breast cancer. In both men and women, colorectal cancer is the second most common cause of cancer death (Commission Regulation (EC) No 401/2006). Over the recent years, the scientific community has been trying to find ever more effective treatments with fewer side effects. However, many studies are still being conducted at this level (Steichen et al., 2013; Voskens et al., 2013).

In order to better understand the biology of cancer and how the tumour growth and metastatic dissemination are processed, Hanahan & Weinberg (2011) proposed six hallmarks of cancer. One of these hallmarks is the ability of tumour cells to resist cell death. Therefore, therapies based on the induction of cancer cell death, namely programmed cell death (PCD), may constitute one approach to help in the treatment of certain types of cancer. Indeed, some drugs that induce apoptosis are already available such as inhibitors of Bcl-2 (*e.g.*, venetoclax; Cang et al., 2015).

Several studies have confirmed the antitumour potential of extracts and compounds obtained from different mushroom species (Ferreira et al., 2010; Giavasis, 2014; Lemieszek & Rzeski, 2012; Lindequist et al., 2005; Poucheret et al., 2006; Zaidman et al., 2005). Thus, the identification and chemical characterization of mushroom extracts that induce PCD in tumour cells may allow the discovery of novel compounds to be used as drugs or nutraceuticals in cancer treatment. In fact, in some countries such as Japan, some mushroom extracts/fractions/compounds are approved to be used in cancer treatments. Some examples are PSP (polysaccharide-peptide) and PSK (polysaccharide-K) (Zaidman et al., 2005), lentinan (polysaccharide; Ina et al., 2013) and schizophyllan (polysaccharide; Sullivan et al., 2006). Therefore, the identification of the compounds responsible for the bioactivity is crucial for the development of nutraceutical and/or pharmaceutical formulations.

The balance between cell division and cell death is very important for the maintenance of multicellular organisms. Given its importance, these processes are highly regulated. It is well recognised that cells have a mechanism of PCD, mediated by an intracellular program, which is established to conduct death of a cell in any pathological state format (Ouyang et al., 2012). Taking into account the phenotypic and molecular factors involved in the process, it is accepted that cells have multiple routes through which they can execute PCD, including apoptosis (the most studied mechanism), autophagy and programmed necrosis (Bialik et al., 2010). These three main mechanisms of PCD are characterized by distinct morphological features and are executed through different signalling pathways (Bialik et al., 2010; Ouyang et al., 2012; Tan et al., 2009).

Apoptosis is characterized by several events such as cell shrinkage, chromatin condensation and fragmentation, membrane blebbing and disintegration of the cell into apoptotic bodies; autophagy unfolds through the appearance of double-membrane cytoplasmic vesicles, the autophagosomes, engulfing bulk cytoplasm and/or cytoplasmic organelles such as mitochondria and endoplasmic reticulum for recycling; and necrosis is defined as non-lysosomal vesiculate degradation, which involves cell swelling, organelle dysfunction and cell lysis (Bialik et al., 2010; Krysko & Vandenabeele, 2009; McCall, 2010; Nishida et al., 2008; Ouyang et al., 2012).

Since homeostasis and cell death are very important processes of morphogenesis, an imbalance in these mechanisms leads to various pathological processes including cancer

(Ouyang et al., 2012; Sun & Peng, 2009; Tan et al., 2009). The ability of chemotherapeutic agents and ionizing radiation to induce PCD in tumour cells constitutes the basis of cancer therapy (Tan et al., 2009). Therefore, it becomes very important to fully understand the pathways involved in the various types of PCD, in order to find potential therapeutic targets and to identify novel compounds that are inducers of PCD.

Figure 9 shows, in a simplified way, the apoptosis pathway, while **Figure 10** summarizes the autophagy process, both considered two main types of PCD.

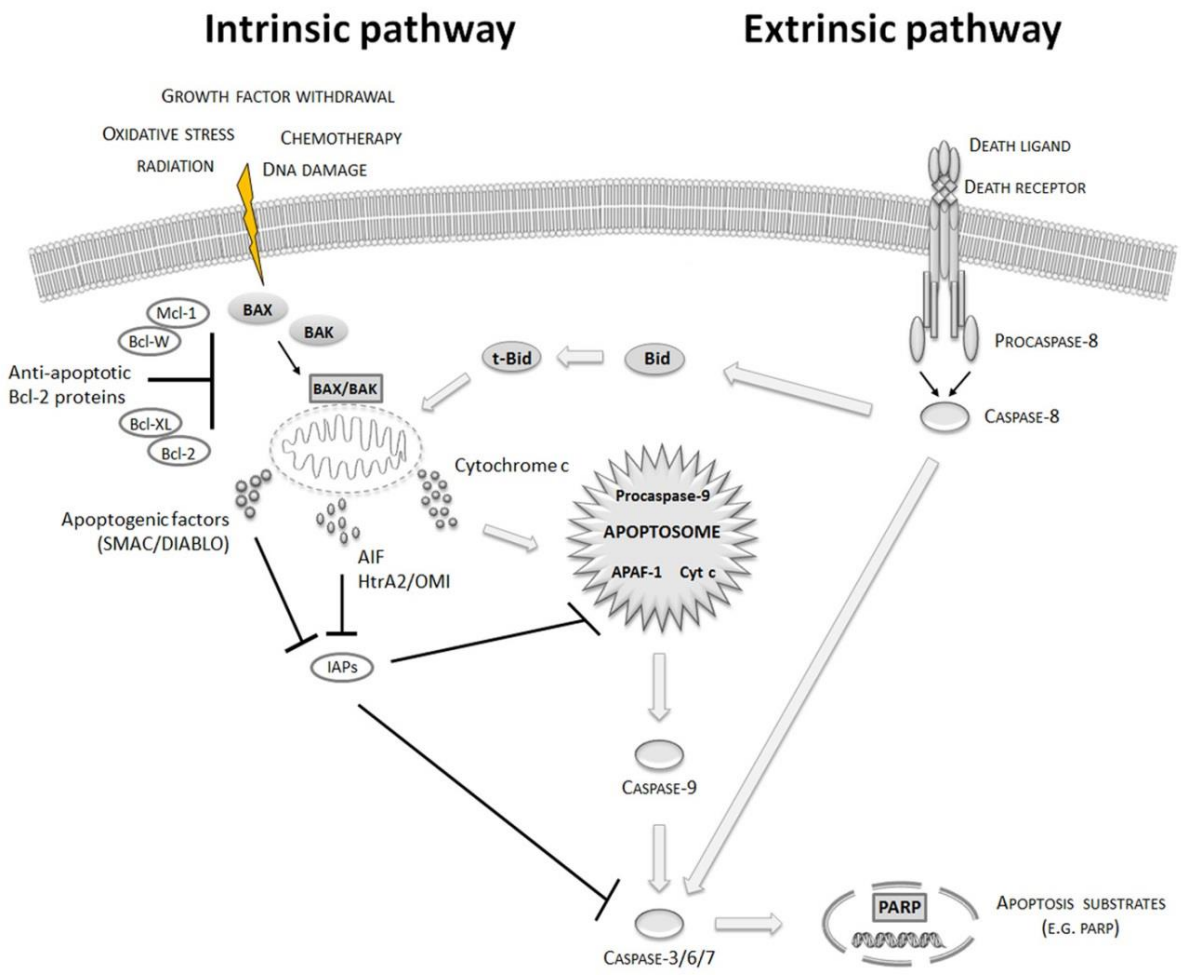


Figure 9. Apoptosis general pathways. In the intrinsic pathway BH3-only proteins (members of the Bcl-2 protein family) induce the oligomerization of Bax or Bak, which triggers mitochondrial release into the cytosol of apoptogenic factors, such as cytochrome c, SMAC/DIABLO and HtrA2/OMI. SMAC/DIABLO and HtrA2/OMI interfere mostly with the caspase-inhibitory function of the inhibitor of apoptosis proteins (IAPs). Once in the cytosol, cytochrome c is recruited into the apoptosome with APAF-1 and procaspase-9, and activates the caspase cascade (initiated with caspase-9 activation). Activated caspase-9 then cleaves and activates the executioner caspases-3, -6 and -7 which will result in the cleavage of death substrates, such as PARP. The extrinsic pathway comprises oligomerization of death receptors (*e.g.*, Fas) with their ligands (*e.g.*, FasL), resulting in the recruitment and activation of the initiating caspase-8 and/or caspase-10. Caspase-8 can directly cleave and activate the executioner caspase-3 (which also belongs to the intrinsic pathway). In addition, activated caspase-8 cleaves Bid (a BH3-only protein member), generating truncated Bid (t-Bid). t-Bid translocation to mitochondria leads to the release of apoptogenic factors, causing further activation of the apoptotic cascade by formation of more apoptosomes (Reis et al., 2016a).

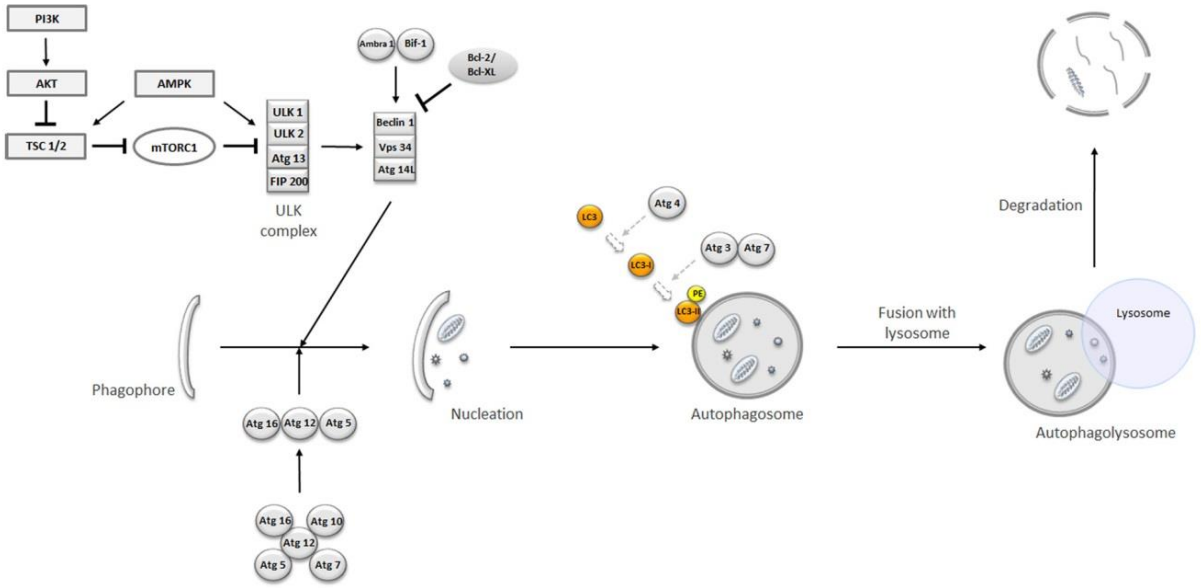


Figure 10. Autophagy general pathways. In the absence of energy/nutrients, AMPK (AMP-activated protein kinase) phosphorylates TSC1/2 activating the ULK kinase complex (ULK 1/2, Atg13 and FIP200) and inhibiting mTOR Ser/Thr kinase. Once mTORC1 kinase (one of mTOR complexes) is inhibited, autophagy is triggered. Beclin 1 binds to VPS 34 (a lipid kinase) following its interaction with other autophagy regulators such as Ambra 1. Autophagy related genes (Atg) such as Atg12, Atg5, Atg7, Atg10 and Atg16, as well as LC3 lipidation into LC3-II, are required for the formation and maturation of the autophagosomes. Concerning the LC3 system, the membrane associated LC3-II is produced after conjugation of LC3-I with phosphatidylethanolamine (PE) through an ubiquitination-like enzymatic reaction. Fusion of the autophagosome with the lysosome results in the digestion and recycling of the autophagolysosomes cargo (Reis et al., 2016a).

Several mushroom extracts have been reported to have influence in PCD, being modulators especially of apoptosis and autophagy in several human tumour cell lines (Reis et al., 2016a). Once again, most of the studies were conducted all over the world. Regarding the Portuguese mycoflora, only the species *Clitocybe alexandri* (Gillet) Gillet (Vaz et al., 2012a), *Suillus collinitus* (Fr.) Kuntze (Vaz et al., 2012b) and *Leccinum vulpinum* Watling (Reis et al., 2016b) were identified as inducers of apoptosis. The *Ganoderma lucidum* (Curtis) P. Karst. species was identified as a modulator of autophagy (Oliveira et al., 2014; Reis et al., 2015).

Part II

Scope and Objectives

//

Parte II.

Ámbito de Aplicación y Objetivos

Scope and Objectives

The Iberian Peninsula, and particularly Bragança (Portugal), is one of the regions of Europe with greater mycological biodiversity, where mushrooms' picking is a tradition. Thus, with this study it is expected to undertake a careful identification and characterization of the largest number of wild mushroom species, contributing to the development of databases and prizing their traditional consumption as foods with high nutritional value.

In addition to its gastronomic and culinary relevance, **the potential** of mushrooms as **functional foods** and as a source of **bioactive compounds** has been explored over time. As aforementioned, since ancient times people have been taking advantage of their medicinal and therapeutic properties, such as antioxidant, anti-cancer, prebiotic, immunomodulating, anti-inflammatory, cardiovascular, antimicrobial, and anti-diabetic, and there is an attempt to promote this natural matrix as a new generation "biotherapeutic".

Given the bioactive/therapeutic importance of mushrooms, there was also a demand for identifying potentially bioactive extracts, testing the antioxidant, antimicrobial and antitumour potential of the studied species. Therefore, this work aims to take a step forward towards classifying mushrooms, namely the studied species, as functional foods.

From this background, the **main objective** of this research work was:

> *The study of edible mushrooms from the Northeast of Portugal performing their chemical characterization (including the evaluation of their nutritional value, biochemical composition, taking into account the presence of nutrients and bioactive compounds) and testing their biological activity, namely antioxidant, antimicrobial and antitumour capacity. Consequently, it is intended to enhance the traditional use and consumption of mushrooms, valuing them as a possible source of functional ingredients and/or nutraceuticals.*

To the accomplishment of the main goal, the following **specific aims** were established:

1. Chemical characterization of the studied species (wild and cultivated mushrooms) through the evaluation of their nutritional value, and the presence of compounds with nutritional and bioactive interest;
2. Evaluation of the biological activity of the mushroom extracts: *in vitro* antioxidant activity, antimicrobial activity against pathogenic bacteria and fungi, and cytotoxicity effects in human tumour cell lines and in non-tumour cells;
3. Attempt to elucidate the antitumoural mechanism of action of the mushroom extracts on human tumour cell lines (effects on cell proliferation, cell cycle arrest and on programmed cell death).

Ámbito de Aplicación y Objetivos

La Península Ibérica, y en particular Bragança (Portugal), es una de las regiones de Europa con mayor biodiversidad micológica, donde existe una arraigada tradición de recolección y consumo de setas. De modo que con este estudio se espera llevar a cabo la caracterización química de algunas de las especies de setas silvestres más valoradas, intentando contribuir al desarrollo de bases de datos y revalorización del consumo tradicional de las mismas por ser alimentos con gran valor nutricional. Además de su importancia gastronómica y culinaria, las setas se conocen y se utilizan desde la antigüedad por sus propiedades medicinales y terapeuticas, por su potencial antioxidante, anticancerígeno, inmunomodulador, anti-inflamatorio, anti-microbiano, hipoglucemiante y prebiótico, de modo que hoy en día existe un marcado interés por promover estas matrices alimentarias como precursores "bioterapéuticos" de nueva generación por su como **alimentos funcionales** y como fuente de **compuestos bioactivos.**

Dada la importancia de las setas desde el punto de vista funcional, hoy en día también se está tratando de identificar extractos de setas potencialmente bioactivos, poniendo a prueba el potencial antioxidante, antimicrobiano y antitumoral de las especies estudiadas. Por lo tanto, este trabajo pretende dar un paso adelante hacia la consideración de las setas objeto de estudio como alimentos funcionales.

Por todo ello, el presente trabajo de investigación tiene como **objetivo principal**:

> *El estudio de las setas comestibles del Noreste de Portugal, mediante la caracterización química (incluyendo la evaluación de su valor nutricional, composición de nutrientes y compuestos bioactivos) y evaluación de su actividad biológica (capacidad antioxidante, antimicrobiana y antitumoral). Con la finalidad de promover el consumo y uso tradicional de estas setas, valorándolas como una posible fuente de ingredientes funcionales y/o nutracéuticos.*

Para ello se plantearon los siguientes **objetivos específicos**:

1. Caracterización química de las especies seleccionadas (silvestres y cultivadas) mediante la evaluación de su valor nutricional y contenido de compuestos bioactivos;
2. Evaluación de la actividad biológica de los extractos de setas seleccionadas: actividad antioxidante *in vitro*; actividad antimicrobiana frente a bacterias y hongos patógenos; y evaluación de la citotoxicidad en líneas celulares tumorales humanas y en células no tumorales;
3. Primera aproximación a la elucidación del mecanismo de acción antitumoral de los extractos de las setas estudiadas en las líneas de células tumorales humanas (efectos sobre la proliferación celular, modulación del ciclo celular y inducción de la muerte celular programada).

Part III

Working Plan

Working Plan

Given the main objective of the present work, the study of edible wild mushrooms from the Northeastern region of Portugal, performing their chemical characterization and testing their biological activity and valuing them as a possible source of functional ingredients and/or nutraceuticals, the following working plan was followed.

Task 1. Mushrooms selection

In order to comply with the proposed objectives, in the first phase, the samples under study were selected. A total of 17 different species of edible mushrooms were selected and studied. Regarding the cultivated species, these were selected according to the season availability in the market and consumer preferences. Regarding the wild species, these were selected considering their availability and suitability for the proposed objectives. This species were collected and identified by researchers of our group with expertise in mycology. Once comparative studies between wild species from Portugal and Serbia (*Suillus granulatus* (L.) Roussel) were also conducted, the same criteria were followed for the Serbian samples. *Cordyceps militaris* (L.) Link was provided by the Department of Applied Biology, Kangwon National University, Chuncheon, Republic of Korea and *Phellinus linteus* (Berkeley & Curtis) Teng) was provided by Amazing Grace Health Industries, Bangkok, Thailand.

Task 2. Chemical characterization of the studied species

For the species under study a detailed chemical characterization was carried out. The nutritional value, nutrient and non-nutrient composition, taking into account biologically active compounds, were evaluated. Therefore, the nutritional value of the samples was assessed based on protein, fat, carbohydrate and ash contents obtained following standard procedures (AOAC, 2012). Energy was calculated according to Regulation (EC) No. 1169/ 2011 of the European Parliament and of the Council, of 25 October 2011, on the provision of food information to consumers.

For the nutritional evaluation, chromatographic determinations of soluble sugars, tocopherols and fatty acids were carried out. For this purpose High Performance Liquid Chromatography (HPLC) coupled to different detectors (soluble sugars, tocopherols) and Gas Chromatography (GC) coupled to a flame ionization detector (FID) (fatty acids) systems were used.
Regarding the non-nutrient composition, phenolic acids and related compounds, as well as other organic acids were assessed. Herein, liquid chromatography systems coupled to different detectors were used.

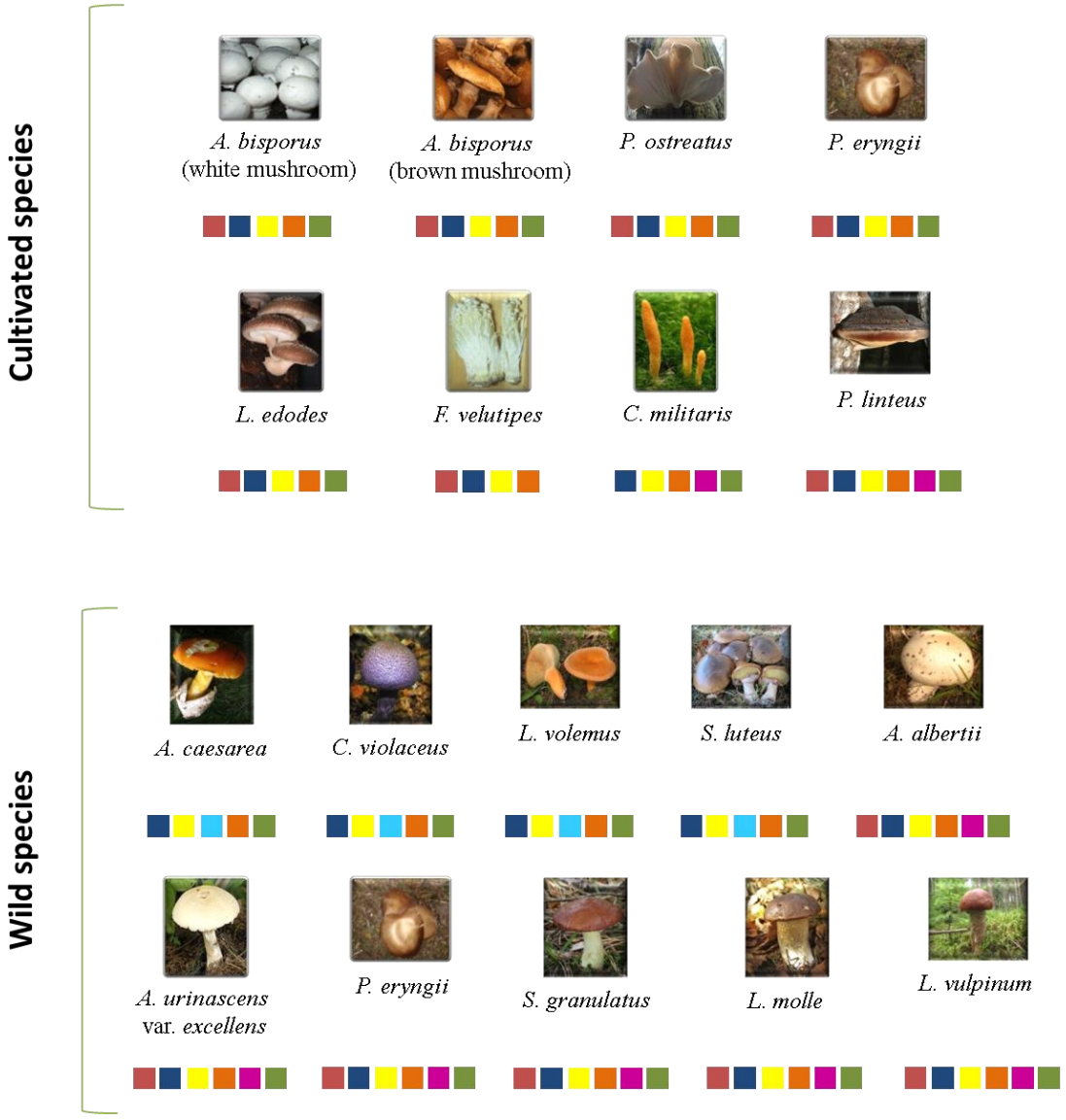
Task 3. Evaluation of the bioactive properties of the studied species

After characterizing the compounds present in the samples, the bioactivity of their extracts was assessed. Thus, the antioxidant, antimicrobial and antitumour potential were evaluated. Given the presence of phenolic acids in all the studied species, especially in the wild samples (the main focus of this study), the methanolic extract of the samples was selected for such evaluations. The antioxidant potential was assessed with different *in vitro* assays; the antimicrobial activity was evaluated testing the extracts against different pathogenic bacteria and fungi; and the antitumour potential verified in different human tumour cell lines. In addition, for some species, it was possible to infer a possible mechanism of action. The safety of the extracts was also evaluated in non-tumour cells, namely in cell cultures obtained from porcine liver (PLP2).

Task 1. Mushrooms selection

Species selected according to the season availability, consumer preferences and suitability for the proposed objectives.

Task 2. Chemical characterization of the studied species



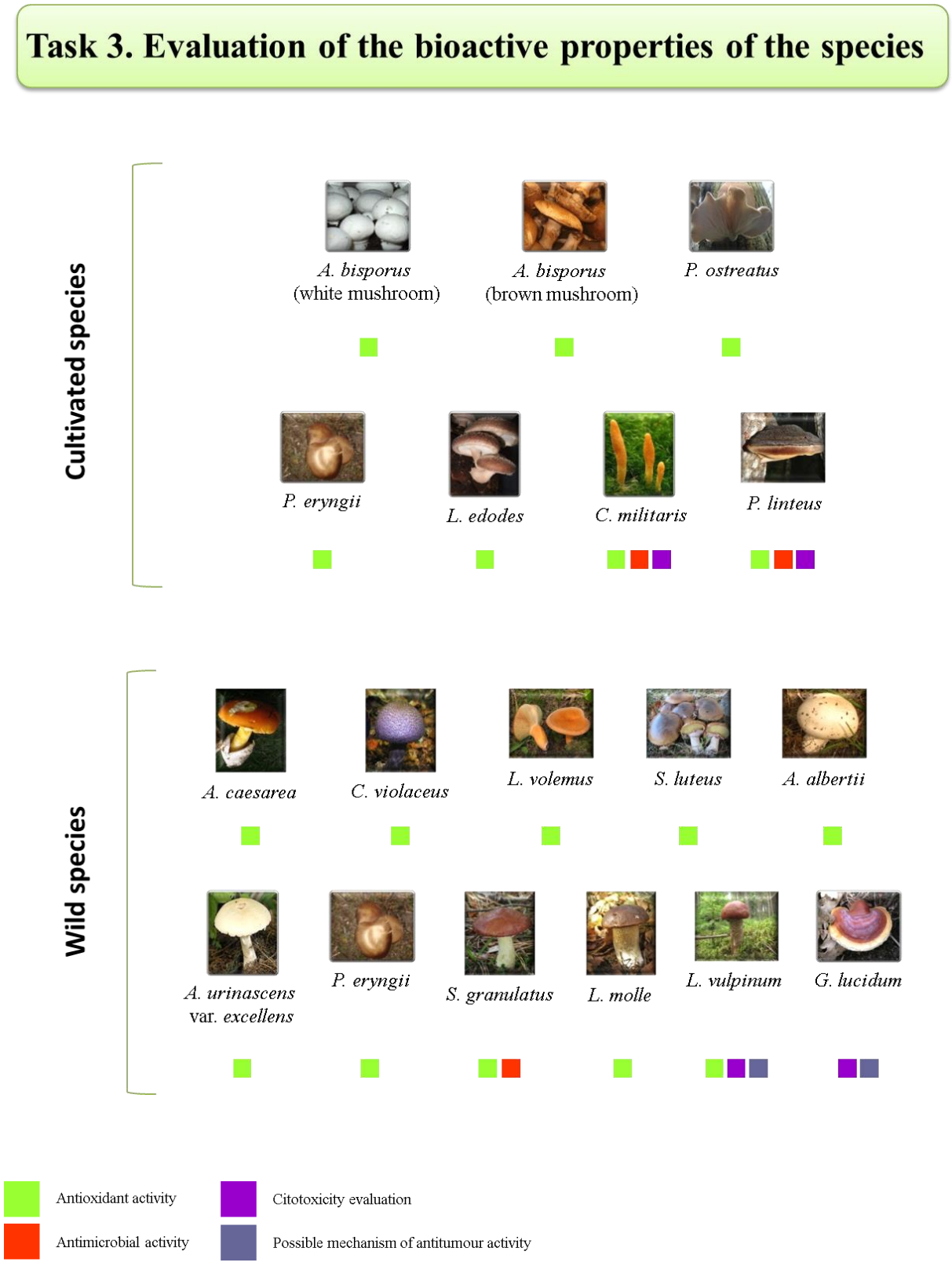


Figure 11. Depiction of the different tasks.

Part IV

Materials and Methods

4.1. Mushrooms: selection and description

Mushrooms have a well-known structure (**Figure 12**). Almost all the mushroom species studied in the present work belong to the phylum Basidiomycota. The only exception was *Cordyceps militaris*, a species belonging to the phylum Ascomycota (Kirk et al., 2008).

The major difference between these two groups resides in their spore-producing structures. Fungi belonging to Basidiomycota phylum produce sexual spores (basidiospores) externally in spore-producing specialized cells – basidia. Basidia are found on the hymenophore (hymenium-bearing structure) of the fruiting bodies. Spores are quit from basidia and then dropped from the gills (or tubes, in boletes case) located underneath the cap (Barceloux, 2012). Spores from Ascomycota phylum (ascospores) are formed inside a tube-like or bag-like structure (ascus). This structure expels the ascospores through a small lid or tip, usually by bursting. That is why sometimes they are called spore-shooters (Barceloux, 2012; Jordan, 2015).

Most of the commercially cultivated mushrooms belong also to the phylum Basidiomycota (Chikthimmah, 2006), and the studied species were not exception. *Agaricus bisporus*, *Lentinula edodes*, *Pleurotus* sp. and *Flammulina velutipes* constitutes the most cultivated species worldwide (Aida et al., 2009; Chang & Miles, 2004). Besides their pleasant flavour, these mushrooms required shorter growth time when compared to other edible mushrooms, claim few environmental controls, and their cultivation is quite simple and cheap (Bonatti et al., 2004).

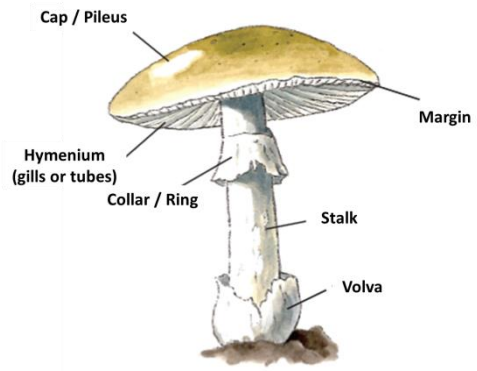


Figure 12. Typical structure of a mushroom (based on Telander (2012), with some alterations).

In the present work, 17 different edible mushroom species were studied (**Table 3**). The general information about each species is presented below.

Table 3. Species selected to carry out the present work.

Scientific name	Origin	Photo	Common name / English name	Ecology	Date of purchase / collection	Studied sample	Country	Studied extract / fraction
Agaricus bisporus (J.E. Lange) Imbach	Cultivated		White button mushroom; Champignon	Saprotrophic	Spring 2011	Fruiting body Mycelium	Portugal	Methanolic
	Cultivated		Brown mushroom; Portobello		Spring 2011	Fruiting body Mycelium	Portugal	Methanolic
Cordyceps militaris (L.) Link	Cultivated		Scarlet caterpillarclub	Entomopathogenic	Autumn 2012	Fruiting body	Korea	Methanolic
Flammulina velutipes (Curtis) Singer	Cultivated		Golden needle mushroom; Velvet shank; Enoki	Saprotrophic	Spring 2011	Fruiting body	Portugal	Methanolic
Lentinula edodes (Berk.) Pegler	Cultivated		Shiitake	Saprotrophic	Spring 2011	Fruiting body Mycelium	Portugal	Methanolic

Table 3 (Cont.)

Scientific name	Origin	Photo	Common name / English name	Ecology	Date of purchase / collection	Studied sample	Country	Studied extract / fraction
Phellinus linteus (Berkeley & Curtis) Teng	Cultivated		Meshima; black hoof mushroom	Parasitic	Autumn 2012	Food supplement	Thailand	Ethanolic Methanolic Fractions: Polysaccharides Glucans Triterpenoids
Pleurotus ostreatus (Jacq. Ex Fr.) P. Kumm.	Cultivated		Oyster mushroom	Saprotrophic	Spring 2011	Fruiting body Mycelium	Portugal	Methanolic
Pleurotus eryngii (DC.) Quél.	Cultivated		King trumpet mushroom; French horn mushroom; king oyster mushroom; king brown mushroom; boletus of the steppes; trumpet royale	Saprotrophic (sometimes parasite on the roots of herbaceous plants)	Spring 2011	Fruiting body Mycelium	Portugal	Methanolic
	Wild				Autumn 2011	Fruiting body Mycelium	Portugal	Methanolic
Agaricus albertii (Bon.)	Wild		Unknown	Saprotrophic	Autumn 2011	Fruiting body	Portugal	Methanolic

Table 3 (Cont.)

Scientific name	Origin	Photo	Common name / English name	Ecology	Date of purchase / collection	Studied sample	Country	Studied extract / fraction
Agaricus urinascens var. *excellens* (F.H. Møller) Nauta	Wild		Macro mushroom	Saprotrophic	Autumn 2011	Fruiting body	Portugal	Methanolic
Amanita caesarea (Scop.) Pers	Wild		Caesar's mushroom	Mycorrhizal	Autumn 2009	Fruiting body	Portugal	Methanolic
Cortinarius violaceus (L.: Fr.) Gray	Wild		Violet webcap	Mycorrhizal	Autumn 2009	Fruiting body	Portugal	Methanolic
Ganoderma lucidum (Curtis) P. Karst.	Wild		Lacquered Bracket; Lingzhi	Saprotrophic; parasitic	Summer 2011	Fruiting body	Portugal	Methanolic
Lactarius volemus (Fr.) Fr.	Wild		Weeping milk cap; Fishy milkcap; Voluminous - latex milky	Mycorrhizal	Autumn 2009	Fruiting body	Portugal	Methanolic

Table 3 (Cont.)

Scientific name	Origin	Photo	Common name / English name	Ecology	Date of purchase / collection	Studied sample	Country	Studied extract / fraction
Leccinum molle Bon (Bon)	Wild		Unknown	Mycorrhizal	Autumn 2012	Fruiting body	Portugal	Methanolic
Leccinum vulpinum Watling	Wild		Foxy bolete	Mycorrhizal	Autumn 2012	Fruiting body	Portugal	Methanolic
Suillus granulatus (L.) Roussel	Wild		Weeping bolete; granulated bolete	Mycorrhizal	Autumn 2012	Fruiting body	Portugal Serbia	Methanolic
Suillus luteus (L.: Fries) Gray	Wild		Slippery jack; Sticky bun	Mycorrhizal	Autumn 2009	Fruiting body	Portugal	Methanolic

Images from Wikipedia or by Filipa Reis

***Agaricus bisporus* (J.E. Lange) Imbach**

Phylum: Basidiomycota;

Class: Agaricomycetes;

Order: Agaricales;

Family: Agaricaceae

A. bisporus, mostly known as champignon (white mushroom) and portobello (brown mushroom), is the most commonly consumed species worldwide, due to its aroma and flavour, both as fresh and canned form. This species is typically cultivated on a compost medium (Chikthimmah, 2006; Lyle, 2010), and thus not so common the consumption of wild species.

Both white and brown mushrooms have similar features. The cap, initially with a square shape, becomes convex to broadly convex, almost flat in mature mushrooms. However, in champignon, the cap's colour is white at first, becoming pale brown with age; and portobello has a cap with a brownish colour, that has throughout its surface some hair like strands that can form tangles with a darker shade. Note that with the advanced state of maturation, these wires may be lost or, in the case of wild species, may not be visible especially after rain. Like other *Agaricus*, in young specimens it can be observed a protective structure (a partial veil) that connects the base of the cap with the stalk. This structure turns out to fade with the fruiting body growth, exposing the gills and allowing the spread of spores (reproductive structures) and the species dissemination (wild species). The gills of both mushrooms are firstly pinkish to pinkish brown, becoming dark brown to blackish with age. The stalk of these mushrooms is white to brownish, smooth or with small scales below the ring (especially in portobello) broader near the base. The ring may disappear with age (or may be absent especially in wild species after rain) (Biswas et al., 2011; McFarland & Mueller, 2009). Wild species usually arises in autumn, single (not in clusters) (Biswas et al., 2011).

Concerning to its consumption, *A. bisporus* is a very versatile species, being consumed in several dishes, raw (*e.g.*, salads) or cooked (*e.g.*, widely used in pizzas or stir fries). It is also incorporated in cream and tomato sauces (Brodeur, 2014).

***Cordyceps militaris* (L.) Link**

Phylum: Ascomycota

Class: Sordariomycetes

Order: Hypocreales

Family: Cordycipitaceae

As previously referred this mushroom belongs to the phylum Ascomycota, so it differs a bit from the others. While Basidiomycota normally have the more traditional mushroom shape (similar to an umbrella), the cap from Ascomycota is similar to a bag.

C. militaris has an orange colour (both cap and stalk) being whitish at the base of the stalk, which emerges single or in clumps from insect pupae buried in the ground (entomopathogenic species).

Usually it arises in late autumn. Although, it is considered unpalatable and is not widely consumed in Europe, its use in Asia (mainly in soups) is mainly due to its pharmacological potential with a known and long history of use in traditional medicine (McKnight & McKnight, 1998).

***Flammulina velutipes* (Curtis) Singer**

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

Family: Physalacriaceae

F. velutipes has both the cap and the stalk very thin (especially the cultivated mushrooms). Both are white to pale brown, and this later is equal in width along the length. It has tiny hairs that provide a velvety texture. The gills are whitish or creamy (Telander, 2012).

The wild mushrooms are a little bigger with a smooth surface golden brown or reddish, being darker in the center. The mushrooms have a brown velvety stalk and yellowish white gills. This saprotrophic species fructifies in late autumn to early spring on old trees or stumps and fallen branches. Both cultivated and wild mushrooms grow in clusters (the stalks are usually

fused with other stalks at the base) (Jordan, 2015; McKnight & McKnight, 1998, Telander, 2012).

This velvet shank mushroom could be used fresh or sautéed. The fresh mushrooms are usually included in salads and when cooked, it is used in stir-fries, soups, stews and sauces (Brodeur, 2014; Jordan, 2015; McKnight & McKnight, 1998; Telander, 2012).

***Lentinula edodes* (Berk.) Pegler**

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

Family: Marasmiaceae

L. edodes, widely known as shiitake, is a widely cultivated and marked species. It is a brown mushroom with whitish gills, with a fibrous texture and woody cream-colored stalks. The wild mushrooms grow especially on oak logs (Brodeur, 2014; Lyle, 2010).

This species is highly appreciated, and known also for its medicinal properties (Bisen et al., 2010; Dai et al., 2015; Israilides et al., 2008).

Shiitake is consumed either fresh as in dried form. It woody stems are usually removed, and this mushroom is typically consumed sautéed and served over wild rice. When used the dried/lyophilized form, the mushroom should be reconstituted in warm water before cooking. Shiitake has a nice consistency and a smoked flavour, such as meat, and can be cooked slowly (Lyle, 2010). It is quite good grilled, and widely used in risottos, pastas, stir fries, fondues, egg dishes, and casseroles (Brodeur, 2014).

***Phellinus linteus* (Berkeley & Curtis) Teng**

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Hymenochaetales

Family: Hymenochaetaceae

P. linteus is widely known for its medicinal properties (Ferreira et al., 2010; Sliva, 2010; Wasser, 2010 and 2011; Wu et al., 2013; Zhu et al., 2008).

It is thick, hard, woody, silky, velvet and it is shaped like a hoof. It has a pale brown to light yellow cap, and the stalk (also stick), ranges from dark brown to black and sometimes is absent. This parasitic fungus is usually found in branches and stems of the host trees, preferentially on mulberry trees (Halpern, 2007; Jais, 2014; Rogers, 2012; Sliva, 2010).

Due to its morphological features and the bitter taste, *P. linteus* is usually consumed as a decoction especially in Korea, China and Japan, to take advantage of its medicinal properties (Halpern, 2007; Rogers, 2012; Sliva, 2010).

***Pleurotus ostreatus* (Jacq. Ex Fr.) P. Kumm.**

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

Family: Pleurotaceae

P. ostreatus has a white to brownish or silvery grey cap, wavy and convex to flattened, rounded down at the margins with irregular size (like an oyster shell). The gills are white to yellowish, descending down to the base. The stalks are minimal (sometimes absent), tough and off-centre (Jordan 2015; Lyle, 2010; Telander, 2012).

Oyster mushrooms are saprotrophic, grow in clusters, and the wild species fructify essentially in autumn on fallen/dead trees or their stumps especially from elm, oak and beech (Jordan 2015; Lyle, 2010; Telander, 2012).

P. ostreatus is marketed and highly sought due to its delicate odour and flavour, and is commonly used in many recipes sliced, sautéed, and made into soup or a casserole. It is a great companion both for meats and fish dishes, and with other mushroom species with stronger flavours. It is also advisable for sauces (Jordan, 2015; Lyle, 2010; Telander, 2012).

***Pleurotus eryngii* (DC.) Quél.**

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

Family: Pleurotaceae

P. eryngii is usually cultivated for consumption and widely appreciated. This species has a convex cap, centrally depressed, with a turning point margin down, and the colour ranges from beige to brown. The gills are whitish or greyish and it has a whitish stalk. Wild mushrooms arise in spring to autumn and, although being reported as saprotrophic species, sometimes they are also parasitic of some species roots (Chang & Hayes, 2013; Phillips, 2013).

It has a very pleasant aroma and flavour, being one of the most appreciated species from this genus and used in various dishes (Chang & Hayes, 2013). Since it has a slight scallop-oyster flavour, it is often used as seafood accompaniment. Actually *P. eryngii* fruiting bodies may give a seafood flavour to the dishes when inserted in sauces and soups. They are also consumed stir-fried (Brodeur, 2014).

***Agaricus albertii* (Bon.)**

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

Family: Agaricaceae

A. albertii has a convex whitish cap with a margin that becomes toothed with age. The gills are grey in young specimens, turning dark brown. The stalk is cream to grey and with a ring very thick and scaly on the upper of the stalk.

Its aroma and taste are pleasant, quite aniseed (Mohamad et al., 2009). As far as we know, there are no uses/recipes available containing this mushroom. However, given its properties, the use should be similar to that of other *Agaricus*.

***Agaricus urinascens* var. *excellens* (F.H. Møller) Nauta**

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

Family: Agaricaceae

This species is similar to another *Agaricus*, namely *Agaricus campestris* (also an edible species), besides it is larger and thicker. The cap has a domed shape and is white, turning a little brown with age. The gills are initially whitish grey, becoming dark brown along the maturation process. This mushroom has also the distinct veil when young, which breaks to reveal the light-coloured gills. Although it can grow in clusters, usually it grows single forming fairy rings, typically in pasture fields in early summer to autumn (Jordan, 2015).
It has a week aniseed odour, which turns more like ammonia with age. Since it has a steady texture, it is good in all mushroom dishes and the young specimens are quite good sliced raw in salads (Jordan, 2015).

***Amanita caesarea* (Scop.) Pers**

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

Family: Amanitaceae

This widely appreciated *Amanita*, known as Caesar's mushroom, is characterized by a convex becoming flattened cap, with a finely striated margin. It has an orange-to yellow colour, darkest in the centre, fading overall with age. The gills are cream or yellowish. The stalk is smooth or with small scales and with the same colour as the cap, and a membranous ring under this. At the base of the mushroom is a distinct volval sac (typically from the genus *Amanita*) (Jordan, 2015; Telander, 2012).
This mycorrhizal species usually appears singly or in fairy rings in pine or oak woodlands during the summer and autumn, especially after summer storms (Jordan, 2015; Telander, 2012).

This mushroom has a pleasant aroma and flavour. For that it could be inserted in many dishes or consumed raw to get the full flavour. Like all mushrooms, but especially this genus, should be consumed very carefully because it includes some deadly species (*i.e.*, *Amanita phalloides*). Actually, it is easily mixed up with *Amanita muscaria*, a hallucinogenic mushroom known as fly agaric.

***Cortinarius violaceus* (L.: Fr.) Gray**

Phylum: Basidiomycota
Class: Agaricomycetes
Order: Agaricales
Family: Cortinariaceae

Violet webcap has a dark violet to dark purple overall (cap and stalk). Its cap is convex, becoming flattened, dry and rough with tiny scales. As some *Agaricus* species, it has a "cortina" developing from the cap margin to the upper stalk (which in fact contributed for the nomination of the genus – *Cortinarius*). Gills have the same colour as the cap and stalk. Its stalk is tough and fibrous, and somewhat enlarged at the base. *C. violaceus* could be found during the autumn, solitary or disseminated on the ground, associated with coniferous trees (Telander, 2012)**.**

Although it is considered edible, the taste is not very appreciated (McKnight & McKnight, 1998). However, it is consumed by the great lovers of mushrooms, especially in soups, stews and stir fries.

***Ganoderma lucidum* (Curtis) P. Karst.**

Phylum: Basidiomycota
Class: Agaricomycetes
Order: Polyporales
Family: Ganodermataceae

Lingzhi mushroom is the most studied medicinal mushroom worldwide (Ferreira et al., 2010; Halpern, 2007; Lai et al., 2010; Wachtel-Galor et al., 2011; Wasser, 2010 and 2011).

The fruiting bodies of *G. lucidum* are kidney shaped bright reddish brown. It has very tiny pores and the stalk, which could be absent, has the same colour of the cap.

G. lucidum arises single or in small clusters, usually as a shelf at the basis of hardwood trees, shrubs, or roots from removed trees, favouring the decaying stumps of chestnut, oak and other broad-leaf trees (Davis et al., 2012).

As *P. linteus*, this species is not consumed as other mushrooms, because of its bitter and woody taste. So, it is used mainly in decoctions and other alternative medicines (*e.g.*, tonics) for medicinal purposes (Halpern, 2007; Davis et al., 2012).

***Lactarius volemus* (Fr.) Fr.**

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

Family: Russulaceae

L. volemus possess a convex cap, depressed in the centre with a flat or incurved margin. It has a uniform reddish-orange colour and a straight, smooth, and pale orange-brown stalk. As all the *Lactarius* species, when cut, the flesh exudes latex (hence the name of the genus). The latex colour should be taken into consideration, because it is white initially and then turns brown; if it turns yellow or lilac, is not advisable its consumption, as it is referred to as poison (McKnight & McKnight, 1998; Ostry et al., 2011; Telander, 2012).

L. volemus is a mycorrhizal species that usually grows in deciduous woodlands during the summer and autumn and can be associated with conifers and hardwood trees (McKnight & McKnight, 1998; Ostry et al., 2011; Telander, 2012). The release of latex should be taken into account in its preparation for consumption. It may be consumed raw, but it is also considered an excellent choice for dishes with grains/seeds, since it has a meaty taste and a firm and fibrous texture (Brill, 2010; Garibay-Orijel et al., 2007).

***Leccinum molle* Bon (Bon)**

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

Family: Boletaceae

Although not very know species, maybe because it was knew for many years as *L. umbrinum* J. Blum, which name cannot be used for nomenclatural reasons (Kibby, 2000). This species has very soft and dented cap, adopting the fingerprint at the lowest pressure. It is brown, grayish with a lemon tonality, ochre and then clearly olive by the margin. The stipe is is grayish beige, becaming reddish or dirty, and yellowish green under the hymenium (tubes), with gray, later reddish lax scales. The flesh is greenish gray, slightly reddish, and yellow green on top of stipe (Courtecuisse & Duhem, 2005). It is usually found in very wet locations under birch (Kibby, 2000).

***Leccinum vulpinum* Watling**

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

Family: Boletaceae

This is also not a much common and known species. It has a dark brick red to purplish chestnut cap, with a porous whitish or pale cream hymenium. The stalk is white or cream, bruising bluish green at the base. This stalk is characterized by possessing some scales, firstly white, turning then the chestnut colour (Ellis & Ellis, 1990).

The foxy bolete is a mycorrhizal species that usually stablish the association with the genus *Pinaceae* and *Bearberry*. It is an edible species and good if adequately cooked (Gry & Andersson, 2014).

***Suillus granulatus* (L.) Roussel**

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Boletales

Family: Suillaceae

The granulated bolete has sticky brown to yellowish cap with a very tinny membranous margin. As other *Suillus*, this cap is shiny when dry. The stem is lemon yellow and the hymenium is composed by yellow tubes (Jordan, 2015). Note that *Suillus* species, as boletes, are important food for many invertebrates such as insect larvae, snails and slugs, and as their hymenium is porous, is quite common to find such animals in the fruiting bodies (Ostry et al., 2011).

This mycorrhizal species appears in summer or autumn, being more common in the latter season. It is usually associated with coniferous trees, appearing scattered or in clusters (Jordan, 2015; McKnight & McKnight, 1998).

S. granulatus has an aroma and taste slender but enjoyable. For consumption, as *Suillus* sp. in general, the removal of the skin of the cap is advisable. Due to its flavour (not too intense), it is appreciated mixed with other mushroom species, being advisable its dehydration first (Jordan, 2015).

***Suillus luteus* (L.: Fries) Gray**

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Boletales

Family: Suillaceae

This species, also known as slippery jack mushroom, own a convex cap becoming flattened, with various shades of red-orange, brown, or yellow. The surface is slimy when wet and shiny even when dry (since it is covered by a kind of mucus). The stalk is thick, yellowish above and smudged purple below, with a membranous collar that is part whitish and part purplish. The hymenium has a spongy surface whitish to yellow, becoming darker with age (Telander,

2012). *S. luteus* usually arises in late summer-autumn and stablish a symbiosis with especially with pine trees, being often found along pathways on the forest edges, single or in goups (Jordan, 2015; McKnight & McKnight, 1998; Ostry et al., 2011; Telander, 2012).
Its edibility is good, so it can be cook on its own or with other *Suillus*. Often, the juice is strained and used to make a sauce. This mushroom softens well when cooked, so is excellent for soups, being advisable to dry the mushrooms first (Jordan, 2015).

4.2. Standards and reagents

4.2.1. For chemical analyses

Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also other individual fatty acid isomers, ascorbic acid, sugars (D(-)-fructose, D(-)-mannitol, D(+)-raffinose pentahydrate, D(+)-trehalose), tocopherols (α-, β-, γ-, and δ-isoforms) standards, phenolic standards (gallic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, protocatechuic acid) and cinnamic acid and all organic acids all organic acids standards (oxalic acid, quinic acid, malic acid, citric acid, and fumaric acid). Racemic tocol, 50 mg/mL, was purchased from Matreya (PA, USA). Methanol and all other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

4.2.2. For bioactivity evaluation

Antioxidant activity assays. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). β-carotene and linoleic acid were acquired from Sigma and Tween 80 from Panreac. All other solvents and reagents were purchased from scientific retailers. Methanol and all other chemicals and solvents were of

analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

Antimicrobial activity assays. Mueller-Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia). Dimethylsulfoxide (DMSO) (Merck KGaA, Germany) was used as a solvent. Streptomycin, ampicillin, ketoconazole and tryptic soy broth (TSB) were acquired from Sigma-Aldrich (St. Louis, MO, USA), bifonazole from Srbolek (Belgrade, Serbia), tween 80 from Sineks (Belgrade, Serbia). Iodonitrotetrazolium chloride (INT) was acquired from Biochemica (Panreac, Barcelona, Spain) and all other chemicals and solvents were purchased from scientific retailers.

Antitumour activity and cytotoxicity assays. Dimethylsulfoxide (DMSO) (Merck KGaA, Germany) was used as a solvent. Fetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsine, EDTA (ethylenediaminetetraacetic acid), nonessential amino acids solution (2 mM), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, USA). Phosphate buffered saline (PBS), acetic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA), Tris, insulin, hydrocortisone, epidermal growth factor (EGF), cholera toxin, and bromodeoxyuridine (5-bromo-2'-deoxyuridine; BrdU) were acquired from Sigma Chemical Co. (Saint Louis, USA). Paraformaldehyde (PFA) was purchased from Panreac, (Barcelona, Spain). The antibodies mouse anti-BrdU, anti-mouse-Ig-FITC and mouse anti-BCl_2 were from Dako (Agilent Technologies, Santa Clara, USA). The antibodies mouse anti-p53, mouse anti-PUMA, mouse anti-Bax, rabbit BClxL, rabbit anti-PARP, rabbit anti-*p*-H2A.X, goat anti-actin, goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP and donkey anti-goat IgG-HRP were purchased from Santa Cruz Biotechnology (Heidelberg, Germany), and the antibody mouse anti-p21 from Calbiochem (Merck KGaA, Germany). The antibodies rabbit anti-caspase and rabbit anti-VPS34, rabbit Beclin-1 and rabbit anti-Light Chain 3 B (LC3) were from Cell Signalling Technology (Danvers, MA, USA). The antibody rabbit anti-p62 was acquired from Enzo (New York, NY, USA). Protease inhibitor cocktail was purchased from Roche Holding AG (Basel, Switzerland), DC™ Protein Assay kit from Bio-Rad (Hercules, CA, USA) and nitrocellulose membranes from Amersham (Pittsburgh,

PA, USA). The Amersham™ ECL Western Blotting Detection Reagents and the Amersham Hyperfilm ECL were purchased from GE Healthcare (Little Chalfont, United Kingdom) and the Kodak GBX developer and fixer from (Sigma-Aldrich, Sintra, Portugal). The lysossomal inhibitor E-64d was purchased from AppliChem (Darmstadt, Germany) and pepstatin A from Cayman Chemical (Ann Arbor, MI, USA). LC3-mCherry was a kind gift from Prof. T. Johansen (Institute of Medical Biology, University of Tromsø, Norway). Lipofectamine was acquired from Invitrogen (Carlsbad, California, United States). Human Annexin V-FITC/PI apoptosis kit was purshased from Bender MedSystems (Vienna, Austria).

4.3. Chemical characterization

As abovementioned, the studied mushroom species were chemically characterized regarding their nutritional value, nutrients and non-nutrients composition, taking into account that some of the survey compounds are known for their bioactive potential (**Figure 13**).

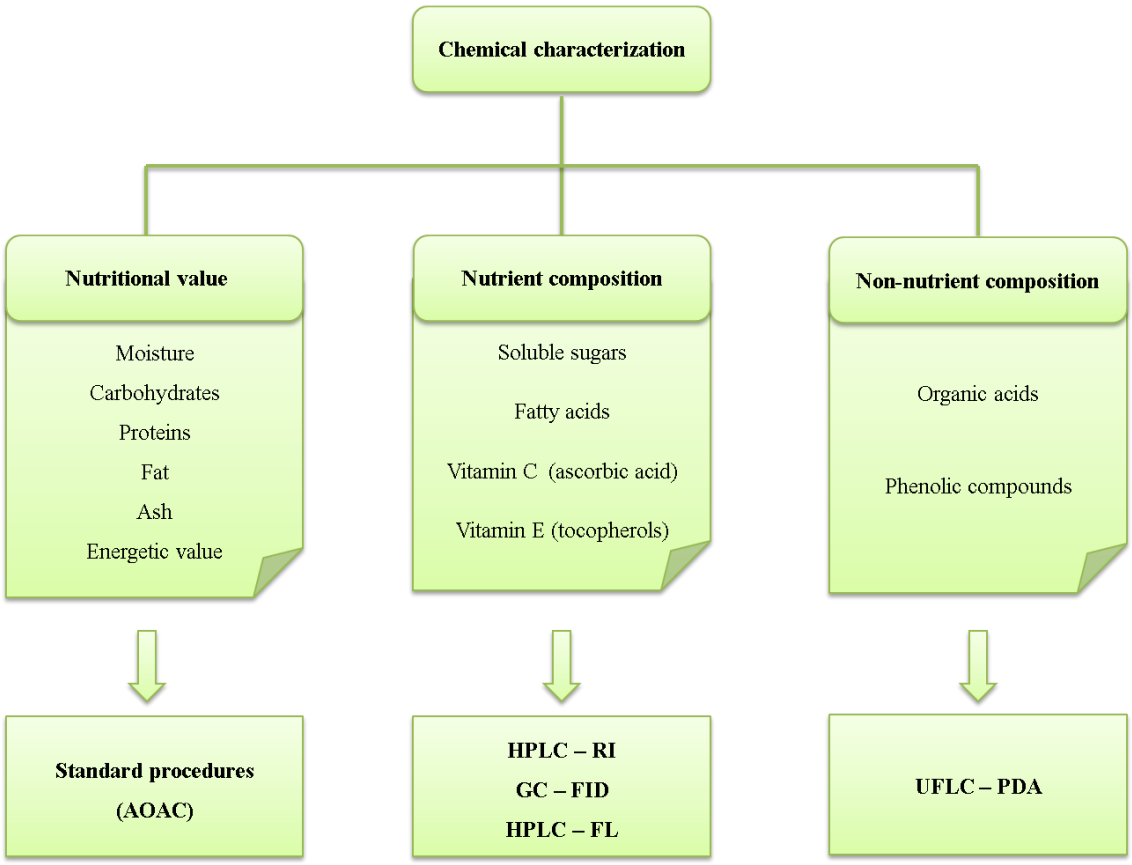


Figure 13. Schematic representation of the evaluated parameters and techniques used to perform the chemical characterization of the mushroom species.

4.3.1. Nutritional value

The samples were analysed for chemical composition (moisture, proteins, fat, carbohydrates and ash) using the AOAC procedures (AOAC, 2012). The crude protein content (N × 4.38) of the samples was estimated by the macro-Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600 ± 15 ºC. Total carbohydrates were calculated by difference. Energy was calculated according to Regulation (EC) No. 1169/2011 of the European Parliament and of the Council, of 25 October 2011, on the Provision of Food Information to Consumers, following the **Equation 7**:

$$\text{Energy (kcal/100g dw)} = 4 \times (g\ protein\ + g\ carbohydrate) + 9 \times (g\ fat)$$

Equation 7. Equation for energy determination.

4.3.2. Nutrient composition

For the nutrient composition, the content in soluble sugars and fatty acids was considered.

Soluble sugars

The lyophilized samples (1 g) were spiked with raffinose as internal standard (IS, 5 mg/mL) and were extracted with 40 mL of 80% aqueous ethanol at 80ºC for 30 min. The resulting suspension was centrifuged at 15,000g for 10 min. The supernatant was concentrated at 60ºC and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40ºC, the solid residues were dissolved in water to a final volume of 5 mL and filtered through 0.2 µm nylon filters for analysis by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI), as previously optimized by Heleno et al. (2009). The HPLC equipment consisted of an integrated system with a Smartline 1000 pump (Knauer, Berlin, Germany), a Smartline manager 5000 degasser, an AS-2057 auto-sampler (Jasco, Easton, MD) and a Smartline 2300 refraction index (RI) detector (Knauer). Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (5 µm, 4.6 × 250 mm, Knauer) operating at 30ºC. The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 mL/min. Sugars identification was made by comparing the relative retention times of sample peaks with standards. Quantification was made by the internal standard method and the results were expressed in g per 100 g of fresh weight (fw) or in g per 100 g of dry weight (dw).

Fatty acids

Before the analysis of fatty acids profile, a trans-esterification procedure was performed, following a procedure previously reported by Reis et al., 2012a. The detection was also made by chromatographic techniques, in this specific case, by Gas Chromatography (GC). The GC equipment was composed by a gas chromatograph (DANI 1000, Contone, Switzerland), equipped with a split/splitless injector and a flame ionization detector (FID). Separation was achieved using a Macherey–Nagel (Düren, Germany) column (50% cyanopropyl-methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 µm df). The oven temperature program was as follows: the initial temperature of the column was 50ºC, held for 2 min, then a 30ºC/min ramp to 125ºC, 5ºC/min ramp to 160ºC, 20ºC/ min ramp to 180ºC, 3ºC/min ramp to 200ºC, 20ºC/min ramp to 220ºC and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50ºC. Split injection (1:40) was carried out at 250ºC. The identification was carried out by comparing the relative retention times of the fatty acid methyl esters (FAME) of the samples with commercial standards. The quantification was made using the Clarity 4.0.1.7 Software (DataApex), being the results expressed as relative percentages of each fatty acid.

4.3.3. Bioactive compounds

For bioactive compounds composition, the content in ascorbic acids (vitamin C), other organic acids, phenolic acids and related compounds, as well as tocopherols (vitamin E) was considered.

Ascorbic acid (Vitamin C)

The lyophilized powder (150 mg) was extracted with metaphosphoric acid (1%, 10 mL) for 45 min at room temperature and filtered through a Whatman No. 4 filter paper. The filtrate (1 mL) was mixed with 2,6-dichloroindophenol (9 mL) and the absorbance was measured within 30 min at 515 nm. The content of ascorbic acid was calculated on the basis of the calibration

curve of authentic L-ascorbic acid (0.006 to 0.1 mg/mL), and the results were expressed in mg per 100 g of dry weight (dw).

For some samples (most recently analysed), the methodology followed was that of the organic acids, once the UFLC system was acquired in the laboratory (please see section 4.3.3.1).

Other organic acids

Organic acids were determined following a procedure previously described by the authors Barros et al. (2013). The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan). Separation was achieved on a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C18 column (5 μm, 250 mm × 4.6 mm i.d.) thermostatted at 35°C. The elution was performed with 3.6 mM sulphuric acid using a flow rate of 0.8 mL min^{-1}. Detection was carried out in a DAD, using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight.

Phenolic acids and related compounds

Phenolic acids and related compounds determination was performed using the system previously described for organic acids. Separation was achieved using a Waters Spherisorb S3 ODS-2 C18 (3 μm, 4.6 mm × 150 mm) column thermostatted at 35ºC. The solvents used were: (A) 0.1% formic acid in water; (B) acetonitrile. The elution gradient established was 10% A to 15% B over 5 min, 15% – 25% A in B over 5 min, 25% – 35% A in B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the PDA using 280 nm as preferred wavelength and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet. The phenolic acids and related compounds were quantified by comparison of the area of their peaks recorded at 280 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in mg per 100 g of dry weight.

Tocopherols (Vitamin E)

Vitamin E isoforms were separated by the HPLC equipment above mentioned and described, according to a procedure previously described by other authors (Heleno et al., 2010). The analysis was performed by coupling with a fluorescence detector (FP-2020; Jasco, Easton, MD, USA), which was programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II (5 µm, 250 × 4.6 mm) normal-phase column from YMCWaters (YMC America, Inc., Allentown, PA, USA) operating at 30ºC. The mobile phase used was a mixture of *n*-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min. The peaks detected were compared with commercial standards, being the quantification based on the fluorescence signal, using the internal standard (tocol) method. The results were expressed in µg per 100 g of fresh weight (fw) or µg per 100 g of dry weight.

4.4. Antioxidant activity

The antioxidant properties of the mushroom species were evaluated according to their reducing power, free radicals scavenging activity and ability to inhibit the lipid peroxidation. There is no a single assay that reveals precisely the mechanism of action of all radical sources or all antioxidants in a complex system (Prior et al., 2005). That is why five different methods were performed (**Figure 14**).

The biological systems are very complex and there are several free radicals: reactive oxygen species (ROS; *e.g.,* O_2•-, HO•, or H_2O_2 and LOOH as non-radicals), and reactive nitrogen species (mainly NO), as well as several oxidant sources (the main is the mitochondria - electron chain transport). Free radical species are very unstable molecules and highly reactive. Actually, they could react with proteins, lipids, carbohydrates and even with DNA (Badarinath et al., 2010; Prior et al., 2005). The sources of antioxidants are also diversified (*i.e.*, enzymes such as superoxide dismutase; large molecules like albumin; small molecules such as ascorbic acid; and some hormones for instance estrogen). Because of all these variations and to the different physicochemical properties of oxidants and antioxidants, the latter may act through different mechanisms/reactions. For example, the same antioxidant may exert its protective effect through the alteration of the GSH metabolism, by quenching

ROS or inhibiting the Ca^{2+} influx, as well as can effectively act on different or specific oxidants. Therefore, and as stated before, no single assay is able to capture the different modes of action of each antioxidant (Badarinath et al., 2010; Gülçin, 2012; Ishige et al., 2001; Prior et al., 2005). In recent times, there has been an attempt to find a method to evaluate the antioxidant ability, yet it has not been an easy task. In addition to the above issues, plus the fact that, whatever method is selected as the ideal to determine the antioxidant capacity of certain food/ingredient, this should evaluate its effect in reaction conditions similar to those that occur *in vivo*, which could be a major limitation for an *in vitro* screening (Badarinath et al., 2010; Magalhães et al., 2008).

In the present work, the used methodologies were reducing power through the *Folin-Ciocalteu* assay and the ferricyanide/Prussian blue assay, DPPH (2,2-diphenil-1-picrilhydrazil) radical scavenging activity, β-carotene bleaching inhibition and inhibition of thiobarbituric reactive species (TBARS) (**Figure 14**).

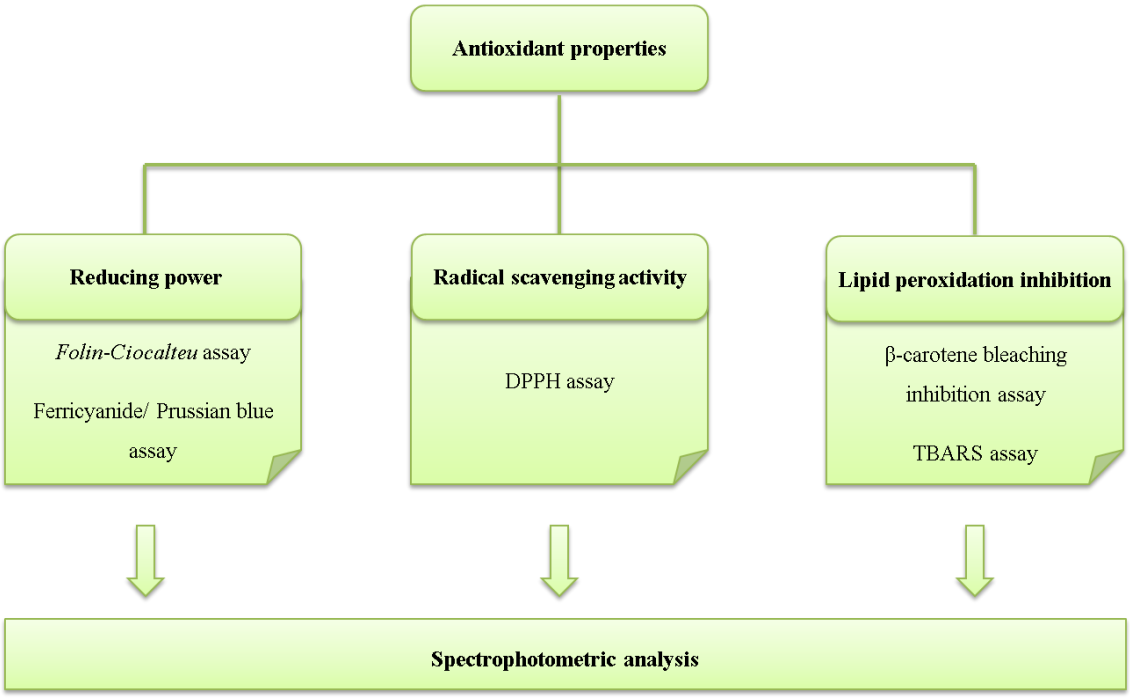


Figure 14. Schematic representation of the assays used to evaluate the antioxidant activity of the mushroom species.

4.4.1. Extracts preparation

The lyophilized samples (1 g) were extracted by stirring with 40 mL of methanol at room temperature for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with an additional portion of methanol (20 mL) for 1 h. The combined methanolic extracts were evaporated under reduced pressure at 40ºC (R-210 rotary evaporator, Büchi, Flawil, Switzerland), re-dissolved in methanol (20 mg/mL) and stored at 4ºC for further use. Sequential dilutions were made from the stock solution (20 mg/mL) and the results are expressed in EC_{50} values, which corresponds to the concentration at the extract is able to inhibit 50% of any oxidative process, involving free radicals or lipid peroxidation (Reis et al., 2012b). EC_{50} values were calculated according the **Equation 8**.

$$EC_{50} = \frac{(x_2 - x_1)}{(y_2 - y_1)} \times (50 - y_1) + x_1$$

Equation 8. Equation for EC_{50} values determination.

Where:

- x_1 is the concentration of the extract whose inhibition percentage is under 50%
- x_2 is the concentration of the extract whose inhibition percentage is over 50%
- y_1 inhibitory percentage under 50%
- y_2 inhibitory percentage over 50%

4.4.2. Reducing power

To evaluate the reducing power of the studied species, two methodologies were performed: *Folin-Ciocalteu* and Ferricyanide/ Prussian blue assays.

Folin-Ciocalteu reagent contains, in its constitution, phosphomolybdic/phosphotungstic acid complexes. The assay is based on the transfer of electrons in alkaline medium from phenolic compounds and other reducing species to molybdenum, forming blue complexes (**Figure 15**) that can be monitored spectrophotometrically at 750–765 nm (Magalhães et al., 2008), according to the following equations:

$$Na_2WO_4 / Na_2MoO_4 \rightarrow (\text{phenol} - MoW_{11}O_{40})^{-4}$$

$$Mo(VI)\ (\text{yellow}) + e^- \rightarrow Mo(V)\ (\text{blue})$$

Equation 9. Two reaction pairs for the reducing power assay.

One of the extract solutions (5 mg/mL; 1 mL) was mixed with the *Folin-Ciocalteu* reagent (5 mL, previously diluted with water 1:10, v/v) and sodium carbonate (75 g/L, 4 mL). The tubes were vortex mixed for 15 s and allowed to stand for 30 min at 40ºC for colour development. Absorbance was then measured at 765 nm (Analytikjena spectrophotometer; Jena, Germany). Gallic acid was used to obtain the standard curve (0.0094 – 0.15 mg/mL), and the reduction of *Folin-Ciocalteu* reagent by the samples was expressed as mg of gallic acid equivalents (GAE) per g of extract.

Figure 15. Example of testing tubes containing solutions after treatment with the *Folin-Ciocalteu* reagent for reducing power determination (photo by F. Reis).

The reducing power assay is based on the ability of phenolics to reduce yellow ferric form to blue ferrous form by the action of electron-donating antioxidants (Benzie et al., 1999). When the antioxidant species is either Fe (III) or $Fe(CN)_6^{3-}$ in the composite ferricyanide reagent, either Fe(II) or $Fe(CN)_6^{4-}$ is formed as the reduction product with the antioxidant, and combines with the other reagent component to produce Prussian blue, $KFe[Fe(CN)_6]$, as the colored product. Thus, when Fe^{3+} is used along with $Fe(CN)_6^{3-}$ as the oxidizing agent, either one of the two reaction pairs occur, both ending up with the same colored product (Berker et al., 2007):

$$Fe^{3+} + \text{antioxidant} \rightleftarrows Fe^{2+} + \text{oxidized antioxidant},$$

$$Fe^{2+} + Fe(CN)_6^{3-} \rightleftarrows Fe[Fe(CN)_6]^-$$

or

$$Fe(CN)_6^{3-} + \text{antioxidant} \rightleftarrows Fe(CN)_6^{4-} + \text{oxidized antioxidant},$$

$$Fe(CN)_6^{4-} + Fe^{3+} \rightleftarrows Fe[Fe(CN)_6]^-$$

Equation 10. Reaction pairs that may occur, allowing to evaluate the reducing power of the samples.

The resulting blue color could be measured spectrophotometrically at 700 nm and it is taken as linearly related to the total reducing capacity of electron-donating antioxidants (Huang et al., 2005) (**Figure 16**).

The extract solutions with different concentrations (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50ºC for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48-wells plate, as also deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm in ELX800 Microplate Reader (Bio-Tek Instruments, Inc; Winooski, United States). The reducing power was obtained directly from the absorbances.

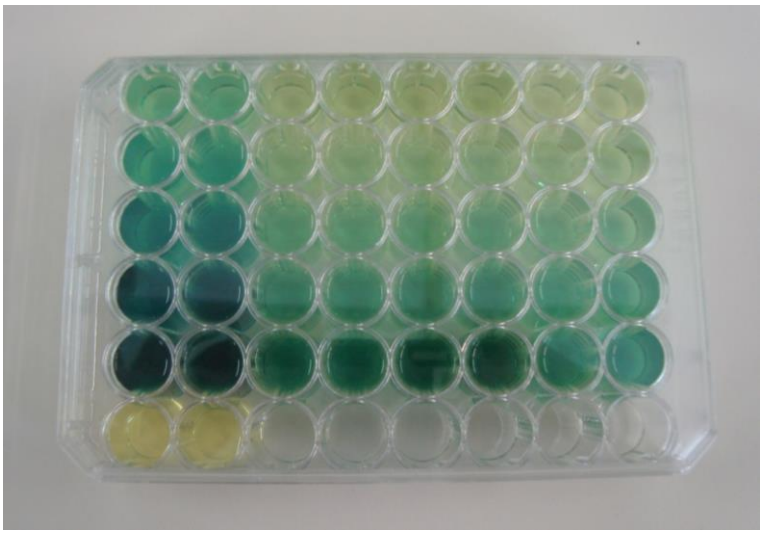


Figure 16. Example of a testing 48-well plate for the evaluation of the reducing power (photo by F. Reis).

4.4.3. DPPH radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical is a longlived organic nitrogen radical with a deep purple color. In the performed method, the purple chromogen radical is reduced by antioxidant / reducing compounds to the corresponding pale yellow hydrazine, according to the following equation:

$$Z^{\cdot} + AH \rightarrow ZH + A^{\cdot}$$

Equation 11. Equation for EC_{50} values determination.

Where:

- $Z^{\bullet}$ represents the DPPH radical
- AH represents the donor molecule
- ZH is the reduced form
- $A^{\bullet}$ is the free radical produced in this first step

This latter radical will then undergo further reactions which control the overall stoichiometry, that is, the number of molecules of DPPH reduced (decolorised) by one molecule of the reductant (Molyneux, 2004). This reduction could be monitored measuring the absorbance decrease at 515-528 nm until the absorbance remains stable in organic media (Karadag et al., 2009), and free radical scavenging activity can be determined by the discoloration of the DPPH solution (Ndhlala et al., 2010) (**Figure 17**).

This methodology was performed using the Microplate Reader mentioned above. The reaction mixture on 96 wells plate consisted of a solution by well of the extract solutions with different concentrations (30 µL) and methanolic solution (270 µL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 30 min in the dark, and the absorption was measured at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation:

$$\% \text{ RSA} = \frac{(A_{DPPH} - A_S)}{A_{DPPH}} \times 100$$

Equation 12. Equation for RSA determination in the DPPH method.

Where A_S is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution.

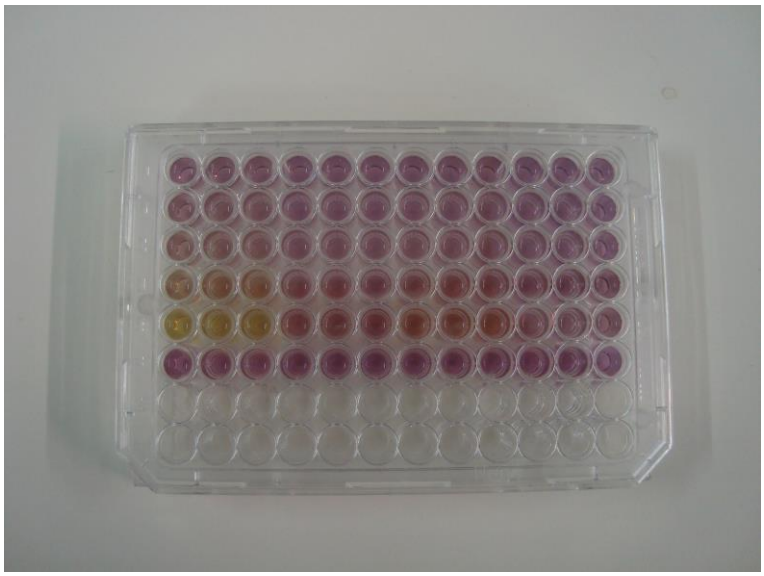


Figure 17. Example of a testing 96-well plate for the evaluation of the DPPH radical scavenging activity (photo by F. Reis).

4.4.4. Lipid peroxidation inhibition

The evaluation of the lipid peroxidation inhibition of the studied species was carried out through two different methods: β-carotene/linoleate (or β-carotene bleaching inhibition assay) and TBARS assays.

In the first method, the antioxidant capacity is determined by measuring the inhibition of the production of volatile organic compounds and the formation of conjugated diene hydroperoxides due to linoleic acid oxidation, which bleach the β-carotene in the emulsion. The reaction mechanism involves the bleaching of carotenoids via heat-induced oxidation and

the resultant discoloration being inhibited or diminished by antioxidants that donate hydrogen atoms to quench radicals (**Figure 18**). Absorbance of β-carotene is measured at 470 nm (Ndhlala et al., 2010). Thus, the reaction mechanism can be described as follows (Kaur & Geetha, 2006):

$$\beta\text{-carotene - H (orange)} + ROOH \rightarrow \beta\text{-carotene}^{\cdot} \text{ (discoloured)} + ROOH$$

$$\beta\text{-carotene - H (orange)} + ROOH + AH \rightarrow \beta\text{-carotene - H (orange)} + ROOH + A^{\cdot}$$

Equation 13. β-carotene bleaching inhibition reaction.

A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40ºC under vacuum and linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into test tubes containing extract solutions with different concentrations (0.2 mL). The tubes were shaken and incubated at 50ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. β-Carotene bleaching inhibition was calculated using the following equation:

$$\beta\text{-carotene bleaching inhibition} = \frac{\beta\text{-carotene content after 2 h of assay}}{\text{initial } \beta\text{-carotene content}} \text{ x } 100$$

Equation 14. Equation of the β-carotene bleaching inhibition assay.

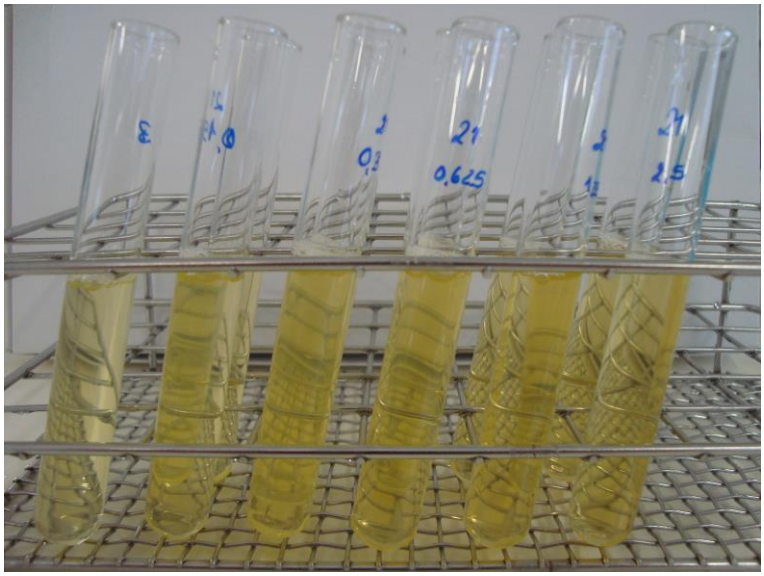


Figure 18. Example of testing tubes for the β-carotene/linoleate assay (photo by F. Reis).

Lipid peroxidation can be determined by the products of the oxidation that react with thiobarbituric acid (TBA) giving rise to pink compounds that are known as thiobarbituric acid reactive species (TBARS). One of the products commonly used as a biomarker of lipid peroxidation is malodialdehyde (MDA) that associated with TBA in the presence of H^+ ions to form a chromogen (MDA-TBA) according to the reaction shown in **Figure 19**. In this methodology, the oxidation of a lipid-rich preparation is induced by addition of a metallic ion (iron or copper), and the extension of the reaction with thiobarbituric acid is determined by the ability of the antioxidants present in the samples to stop the oxidation process, thus inhibiting the formation of the chromogen (**Figure 20**) (Gutteridge, 1995; Kaur & Geetha, 2006; Ng et al., 2000).

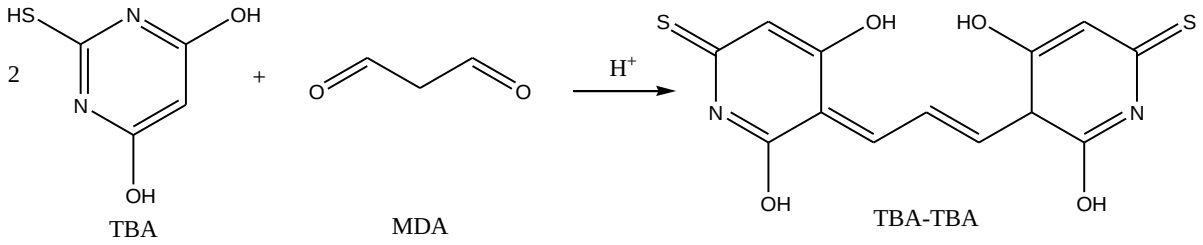


Figure 19. TBARS assay reaction, with the formation of the complex MDA-TBA.

Porcine (*Sus scrofa*) brains were obtained from official slaughtering animals, dissected, and homogenized in ice cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 w/v brain tissue homogenate which was centrifuged at 3000g for 10 min. An aliquot (100 µL) of the supernatant was incubated with the different concentrations of the samples solutions (200 µL) in the presence of $FeSO_4$ (10 mM; 100 µL) and ascorbic acid (0.1 mM; 100 µL) at 37ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 500 µL), followed by thiobarbituric acid (TBA, 2%, w/v, 380 µL), and the mixture was then heated at 80ºC for 20 min. After centrifugation at 3000g for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula:

$$\text{Inhibition ratio (\%)} = \frac{(A-B)}{A} \times 100$$

Equation 15. Equation of the inhibition ratio determination in TBARS assay.

Where A and B were the absorbance of the control and the sample solution, respectively.

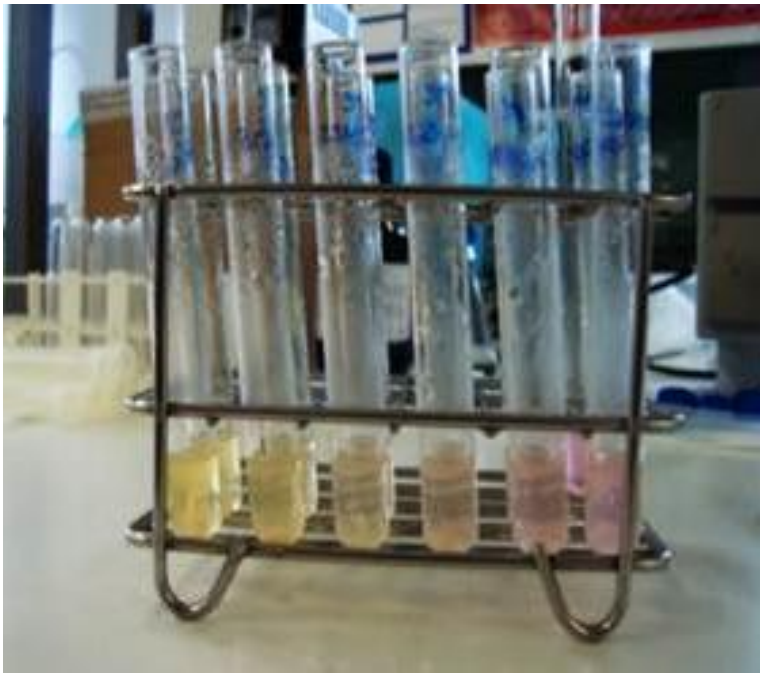


Figure 20. Example of testing tubes for the TBARS assay (photo by F. Reis).

4.5. Antimicrobial activity

To evaluate the antimicrobial activity of the edible mushrooms, different Gram-positive and Gram-negative bacteria were used, as well as different strains of pathogenic fungi, in order to verify whether they inhibited their growth (**Figure 21**).

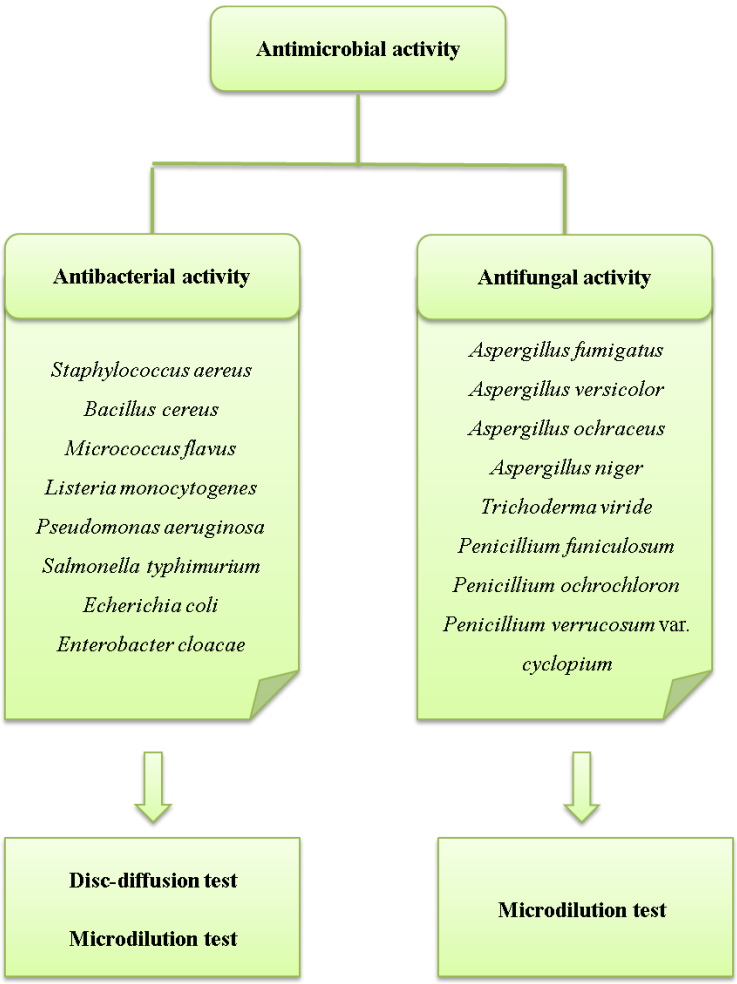


Figure 21. Schematic representation of the assays used to evaluate the antimicrobial activity of the mushroom species.

The antimicrobial activity was carried out to verify if the extracts had any activity towards some food contaminants. Therefore, both the antibacterial and the antifungal activity were evaluated. For each one, the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) were calculated.

4.5.1. Antibacterial activity

For the antibacterial activity determination, the following Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853*), Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030), and Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973) were used. The microorganisms were obtained from the Mycological laboratory, Department of Plant Physiology, Institute for biological research "Sinisa Stanković", University of Belgrade, Serbia.

The assay was carried out in 96 well plates (**Figure 22**), and the minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined by the microdilution method (Espinel-Ingroff, 2001). Briefly, fresh overnight culture of bacteria was adjusted by the spectrophotometer to a concentration of 1×10^5 CFU/mL. The requested CFU/mL corresponded to a bacterial suspension determined in a spectrophotometer at 625 nm. Dilutions of inocula were cultured on solid medium to verify the absence of contamination and check the validity of the inoculum. Different solvent dilutions of methanolic extract were were pipetted into the wells containing 100 µL of Tryptic Soy Broth (TSB) and afterwards, 10 µL of inoculum was added to all the wells. The microplates were incubated for 24 h at 37°C. The MIC of the samples was detected following the addition of 40 µL of iodonitrotetrazolium chloride (INT) (0.2 mg/mL) and incubation at 37°C for 30 min. The lowest concentration that produced a significant inhibition (around 50%) of the growth of the bacteria in comparison with the positive control was identified as the MIC. The minimum inhibitory concentrations (MIC's) obtained from the susceptibility testing of various bacteria to tested extract were determined also by a colorimetric microbial viability assay based on reduction of a INT color and compared with positive control for each bacterial strains (CSLI, 2006; Tsukatani et al., 2012). MBC was determined by serial sub-cultivation of 10 µL into microplates containing 100 µL of TSB. The lowest concentration that shows no growth after this sub-culturing was read as the MBC. Standard drugs, namely streptomycin and ampicillin were used as positive controls. 5% DMSO was used as negative control.

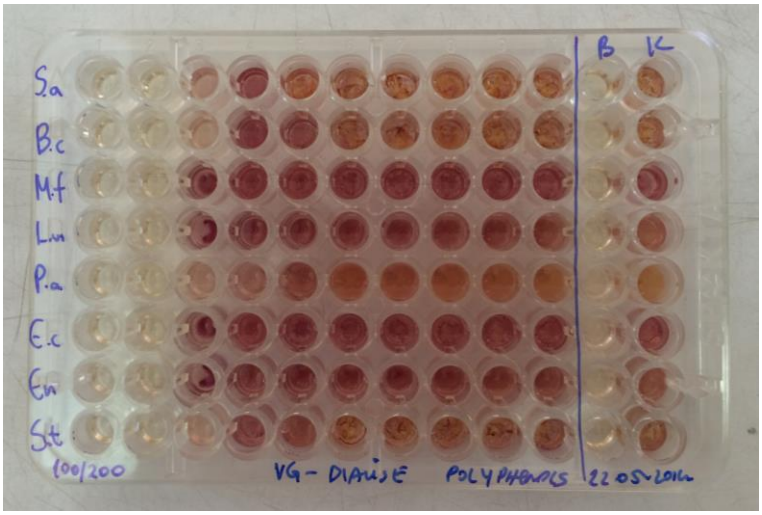


Figure 22. Example of a testing 96 well plate for MIC and MBC determinations (photo by S. Heleno).

4.5.2. Antifungal activity

For the antifungal bioassays, the following microfungi were used: *Aspergillus fumigatus* (1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112) and *Penicillium verrucosum* var. *cyclopium* (strain). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković´", Belgrade, Serbia. The micromycetes were maintained on malt agar (MA) and the cultures were stored at 4°C and sub-cultured once a month (Booth, 1971).

The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 µL/well. The inocula were stored at 4°C for further use. Dilutions of the inocula were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculum.

Minimum inhibitory concentrations (MIC's) determination was performed by a serial dilution technique using 96-well microtitre plates (**Figure 23**). The investigated extract was dissolved in 5% solution of DMSO and added to broth malt medium with fungal inoculum. The

microplates were incubated for 72 h at 28°C. The lowest concentrations without visible growth (at the binocular microscope) were defined as MIC. The minimum fungicidal concentrations (MFCs) were determined by serial subcultivation of 2 µL in microtitre plates containing 100 µL of malt broth per well and further incubation for 72h at 28°C. The lowest concentration with no visible growth was defined as the MFC, indicating 99.5% killing of the original inoculum. 5 % DMSO was used as a negative control, while bionazole and ketokonazole were used as positive controls.

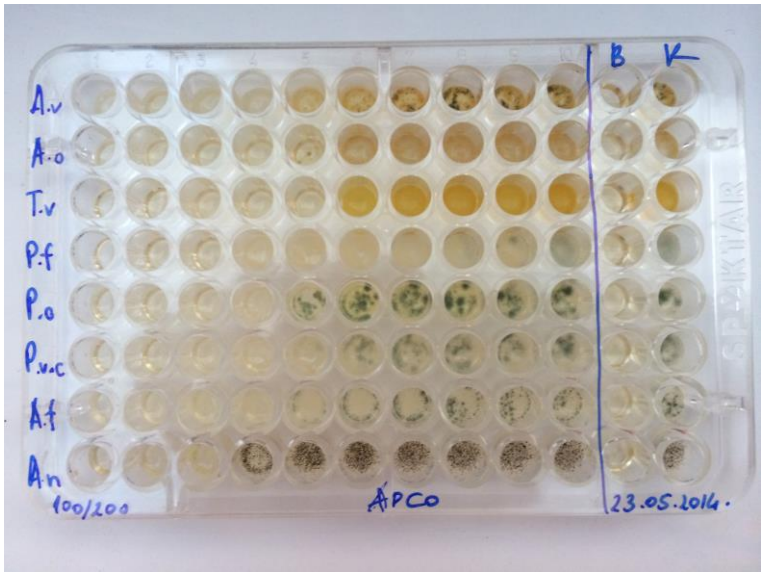


Figure 23. Example of a testing 96 well plate for MIC and MFC determinations (photo by S. Heleno).

4.6. Antitumour activity

4.6.1. Citotoxicity evaluation

Despite the small number of human trials for mushrooms consumption, there is evidence that mushrooms, as well as their fractions, are usually well tolerated with few, or any, side effects (Roupas et al., 2012). However, to initiate studies of the safety of the studied extracts for therapeutic uses, their hepatotoxicity was evaluated on a primary culture of porcine liver cells,

designed as PLP2 (Abreu et al., 2011). It is important to remember that no *in vitro* assay is able to measure the toxicity and particularly hepatotoxicity by itself. Nevertheless, it is always important to perform some assay, using models similar to human in terms of cellular and physiological function, providing preliminary data (Abreu et al., 2011). The effects of the extracts on the cell growth were evaluated according to the procedure adopted in the NCI's *in vitro* anticancer drug screen, which uses the sulforhodamine B (SRB) assay to assess cell growth inhibition (Monks et al., 1991; Vichai & Kirtikara, 2006). This colorimetric assay estimates the cell number indirectly, by staining cellular protein with SRB, bright-pink aminoxanthene dye which binds to cell protein components. It can be spectrophotometrically measured at 515 nm. It is possible to infer that an increased amount of protein produced by cells (as they divide), will correspond to an increase in O.D. at 515 nm.

A screening of the growth inhibitory activity in human tumour cell lines was performed. For that, the procedure referred above for the citotoxicity effects (SRB assay) was followed (**Figure 24**).

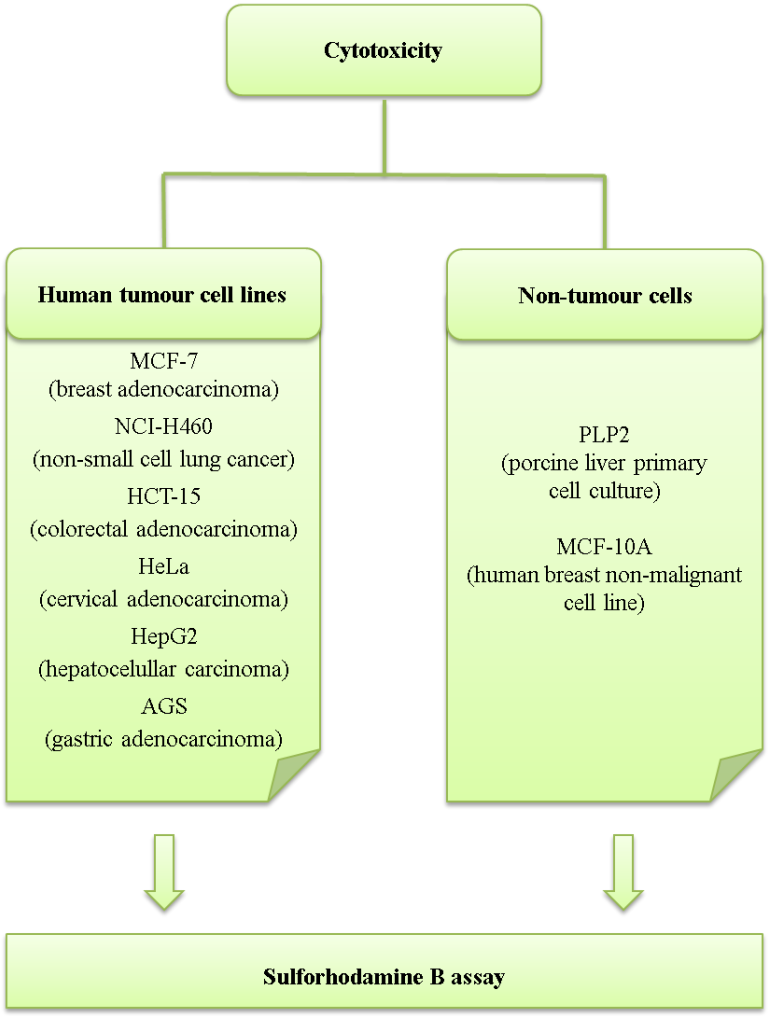


Figure 24. Schematic representation of the assays used to evaluate the citotoxicity activity of the mushroom species.

The extracts and fractions were re-dissolved in different solvents (including water or DMSO) at different stock concentrations. Successive dilutions were made from the stock solution and tested against different human tumour cell lines: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HCT-15 (colorectal adenocarcinoma), HeLa (cervical adenocarcinoma), HepG2 (hepatocellular carcinoma) and AGS (gastric adenocarcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS (MCF-7, NCI-H460, HCT-15 and AGS) and 2 mM glutamine or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (HeLa and HepG2 cells) (Reis et al., 2013, Reis et al., 2016b). Cells were plated in 96-well plates at an appropriate density (5.0×10^3 cells/well for MCF-7 and NCI-H460; 1.0

$\times 10^4$ cells/well for HCT-15, HeLa and HepG2; and 7.5×10^3 cells/well for AGS) and treated for 48 h with the diluted extracts/fractions solutions (**Figure 25**). Following 48 h of incubation with the extract/fraction (or immediately for the T_0 plate), plates were fixed by adding ice-cold 10% trichloroacetic acid (w/v, final concentration) and incubated for 60 min at 4ºC. Plates were then washed with deionized water and dried; sulforhodamine B solution was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried, and the bound dye was solubilised by adding 10 mM Tris base and the absorbance was measured at 510 – 540 nm (Monks et al., 1991). The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth). Ellipticine, doxorubicin or etoposide were used as positive control.

For the possible hepatotoxicity evaluation, a hepatic cell, designed as PLP2, was used (Abreu et al., 2011). PLP2 cells were cultured and maintained in DMEM supplemented with 10% heat-inactivated FBS, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin (Reis et al., 2013). Cells were plated in 96-well plates at an appropriate density (1.0×10^4 cells/well) and treated for 48 h with the different diluted sample solutions. The same procedure described above for the SRB assay was followed. The results were expressed in GI_{50} values. Ellipticine was used as positive control.

Some extracts, were also tested on a human breast non-malignant cell line, designed as MCF-10A. MCF-10A cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM): F12, supplemented with 5% heat inactivated horse serum, 10 mg/mL insulin, 0.5 mg/mL hydrocortisone, 20 ng/mL epidermal growth factor (EGF), and 100 ng/mL cholera toxin (Reis et al., 2016b). Cells were plated in 96-well plates at an appropriate density (5.0×10^3 cells/well) and treated for 48 h with the different diluted sample solutions. The same procedure described above for the SRB assay was performed. The results were also expressed as GI_{50} values and doxorubicin was used as a positive control.

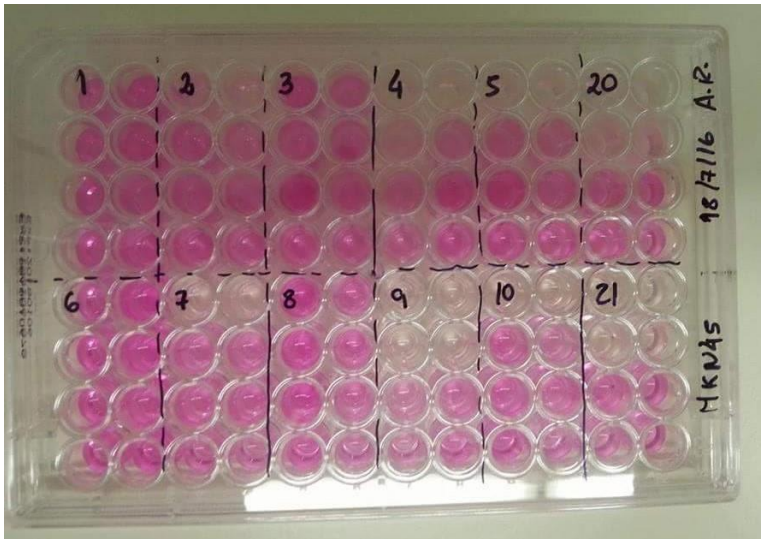


Figure 25. Example of a testing 96 well plate for cytotoxicity evaluation (photo by A. Ribeiro)

4.6.2. Elucidation of the possible mechanism of action

Mushroom extracts/compounds may affect tumour cell growth by different mechanisms. Hence, the mechanism of action of the studied extracts was further studied with different assays. It was sought to determine whether those extracts affected cell proliferation (checking levels of DNA synthesis), caused cell cycle arrest or induced programmed cell death (studying essentially apoptosis and autophagy) (**Figure 26**).

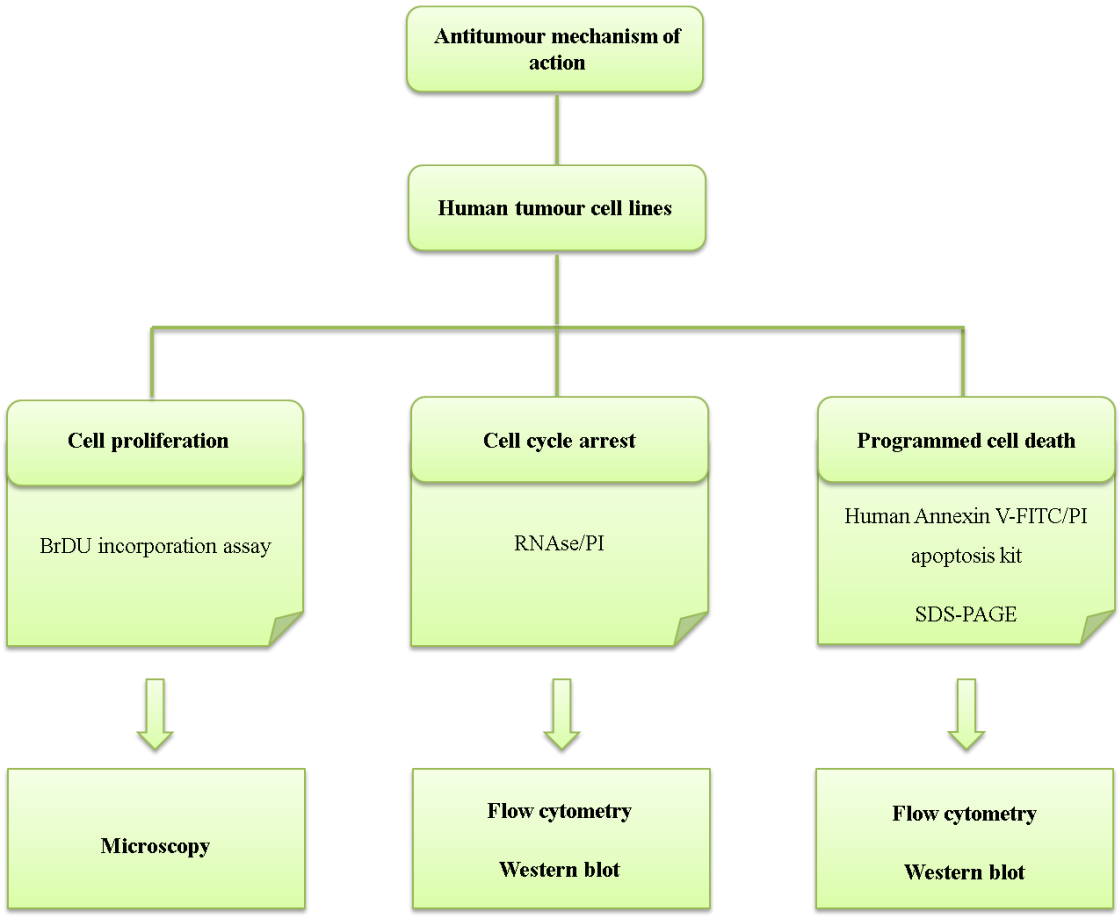


Figure 26. Schematic representation of the assays used to infer the possible mechanism of antitumour activity of the mushroom species.

For these assays, cells were plated on 6-well plates in the following concentrations: 1.0×10^5 cells/well for MCF-7 cells and 1.5×10^5 cells/well for AGS cells, and incubated for 24 h to adhere to the plates. Cells were then treated with the mushroom extracts, at their GI_{50}, GI_{75} or $2 \times GI_{50}$ concentrations.

Cell proliferation analysis

Cell proliferation was analysed with the BrdU (bromodeoxyuridine) assay, according to the protocol previously described by Palmeira et al. (2010). Cells were treated for 48 h with the different concentrations of the extract and one hour before harvesting, cells were incubated with 10 mM BrdU. Afterwards, cells harvested and fixed in 4% paraformaldehyde in PBS for

30 min at room temperature. After centrifugation, cell pellets were re-suspended in PBS and stored at 4ºC. Cell cytospins were prepared and incubated in 2M HCl for 20 min. Following incubation with mouse anti-BrdU antibody for 1 h at room temperature, cells were incubated with anti-mouse-Ig-FITC for 30 min at room temperature. Slides were then prepared with Vectashield mounting medium (with DAPI). BrdU incorporation was observed in a DM2000 microscope (LEICA; Wetzlar, Germany) and a semi-quantitative evaluation was carried out by counting a minimum of 500 cells per slide.

Cell cycle distribution analysis

Following the 48 h treatments, cells were harvested and fixed with ice-cold 70% ethanol and stored at 4ºC for at least 12 h, until further analysis. Cells were re-suspended in PBS containing RNase A (0.1 mg/mL) and propidium iodide (5 mg/mL). Cellular DNA content was analysed by flow cytometry (BD Accuri™ C6 Flow cytometer, USA) and the percentage of cells in the G1, S and G2/M phases of the cell cycle (as well as the percentage of cells in the sub-G1 peak) was determined using the FlowJo 7.6.5 software (Tree Star, Inc., Ashland, OR, USA) after cell debris and aggregates exclusion and plotting at least 15 000 events per sample (Vasconcelos et al., 2000).

Apoptosis analysis

Induced apoptosis was assayed by Flow cytometry using the Human Annexin V-FITC/PI apoptosis kit, according to the manufacturer's instructions, as previously described (Queiroz et al., 2013). Flow cytometry was carried out using the flow cytometer mentioned above and plotting at least 15 000 events per sample. Data were analysed using the BD Accuri C6 Software (version 1.0.264.21).

Autophagy analysis

Transfection with LC3-mCherry expression vector. Cells were plated in 24-well plates and allowed to adhere for 24 h. Transfection with the vector LC3-mCherry was then carried out

using lipofectamine according to manufacturer's instructions. As described by Birame et al. (2012) during the initial 4 h of transfection, cells were incubated with medium containing 5% FBS, which was then replaced by medium containing 10% FBS. Following 24 h of transfection, medium was removed and cells were treated for 48 h with the mushroom extract (*G. lucidum*) (at GI_{50} concentration and 2 × GI_{50} concentration; previously determined with the SRB assay). Cells were then fixed in 4% paraformaldehyde in PBS and analyzed in a fluorescence microscope (Axio Imager.Z1 coupled with ApoTome Imaging System microscope, Zeiss, Oberkochen, Germany).

Treatment with autophagy inhibitors E-64d/Pepstatin. Cells were plated in 6-well plates and allowed to adhere for 24 h. Cells were then treated for 1 h with 15 μg/mL of the lysossomal inhibitors E-64d and Pepstatin A and then co-incubated for 48 h with the GI_{50} and 2 × GI_{50} concentrations of the studied extract. Protein expression analysis was carried out by Western blot, as described below.

Protein expression analysis

For analysis of protein expression, following 48 h of treatments, cell pellets were lysed in Winman's buffer (1% NP-40, 0.1 M Tris – HCl pH 8.0, 0.15M NaCl and 5 mM EDTA) complemented with protease inhibitor cocktail. The total protein content was quantified using the DC™ Protein Assay kit, according to manufacturer's instructions. Proteins (20 mg) were loaded on 12% SDS-PAGE gel and transferred into a nitrocellulose membrane. Different primary and secondary antibodies were used, according with the objectives (survey proteins involved in apoptosis or autophagy). The Amersham™ ECL Western Blotting Detection Reagents, the Amersham Hyperfilm ECL and the Kodak GBX developer and fixer were used for signal detection, as previously described (Lima et al., 2006).

Analysis of DNA damage

DNA damage was analysed with the Alkaline Comet Assay 24 h after treatment with the tested extract. Hydrogen peroxide was used as positive control (1 mM) and was added 10 min

before cells harvesting. Harvested cells were stored in 10% DMSO in FBS at -80ºC, following a procedure previously described by other authors (Seca et al., 2014). After thawing, cells were washed with PBS, re-suspended in 0.6% low melting point agarose and quickly poured onto slides, precoated with 1% agarose in water, with the aid of a coverslip. Slides were kept 10 min on ice. After removing the coverslip, cells were lysed with ice-cold lysis buffer (100 mM Na_2EDTA, 2.5 M NaCl, 10 mM Tris – HCl, pH 10.0, 1% Triton X-100) for 2 h, in the dark, at 4ºC, and then washed twice in ice-cold distilled water for 10 min. Slides were placed on an electrophoresis tank filled with electrophoresis buffer (10M NaOH; 200 mM Na_2EDTA) and incubated for 20 min to let DNA to unwind. Electrophoresis was carried out for 20 min at 23 V, 300 mA. After this period, slides were flooded with neutralisation buffer (0.4 M Tris, pH 7.5) for 20 min and then rinsed in ice cold water for 10 min before being allowed to dry at room temperature overnight. Slides were rehydrated with distilled water (for 30 min), covered with 2.5 mg/mL of propidium iodide solution and incubated for 20 min at room temperature in the dark. Propidium iodide was rinsed with distilled water for 30 min and slides dried at 37ºC. Comets were visualized using the fluorescent microscope mentioned above and representative photographs were taken. The analysis was made using the 'Comet Assay IV v4.3' imaging system (Perceptive Instruments). A minimum of 100 cells per condition were analysed.

4.7. Statistical analysis

The results obtained throughout the different evaluation studies were analysed by applying different statistical tools, selected according to the results and considering the defined research purposes. The software used was Statistics Package for Social Sciences (SPSS) version 22 (IBM Corporation, New York, USA). All results were expressed as mean values ± standard deviations (SD) or standard errors (SE), maintaining the decimal places allowed by the magnitude of standard deviation. All tests were performed at a 5% significance level. For all methods, three samples were mixed to have a representative pool and all assays were carried out in triplicate.

Analysis of variance (ANOVA). Analysis of variance is the most effective method available for analysing more complex data sets (Armstrong & Hilton, 2014). Prior to carrying out the ANOVA test, some preliminary assumptions were verified, namely the homoscedasticity of variances by applying the Levene's test); the normality of distributions (tested by means of the Shapiro Wilks' test); and the existence of statistically significant differences (through the Welch's statistics). The ANOVA results were classified according to the results obtained in the previous tests. In the cases where statistical significance differences were identified, the dependent variable were compared using Tukey's honestly significant difference (HSD) or Games-Howell test, when homoscedasticity was verified or not, respectively. The former applied for homoscedastic samples and the latter for non-homoscedastic samples.

When a specific factor was studied using only two levels, a simple student's t-test was used to classify the results.

Part V

Results

Article 1: Chemical composition and nutritional value of the most widely appreciated cultivated mushrooms: An inter-species comparative study

Reference: Filipa S. Reis, Lillian Barros, Anabela Martins, Isabel C.F.R. Ferreira. (2012). *Food and Chemical Toxicology*, 50, 191-197.
DOI: 10.1016/j.fct.2011.10.056

Resumen

En este trabajo, se analizó y comparó la composición química y el valor nutricional de algunas de las especies de setas cultivadas más consumidas: *Agaricus bisporus* (champiñón común y portobello), *Pleurotus ostreatus* (gírgola o champiñón ostra), *Pleurotus eryngii* (seta de cardo), *Lentinula edodes* (shiitake) y *Flammulina velutipes* (seta de aguja de oro). La seta Shiitake presentó los valores más altos de macronutrientes, a excepción de las proteínas, así como los contenidos más elevados de azúcares solubles, tocoferoles y de AGPI, y los más bajos para los AGS. Los champiñones (común y portobello) mostraron una composición similar de macronutrientes, así como valores similares de hidratos de carbono totales, AGMI, AGPI y tocoferoles. Mientras que el champiñón ostra y la seta de cardo fueron las que presentaron los contenidos más altos de AGMI, con contenidos similares en AGPI, AGMI y AGS, en ambas muestras. También presentarón valores similares de humedad, cenizas, hidratos de carbono y energía. Este estudio contribuye a la elaboración de bases de datos nutricionales de algunas de las especies de setas más consumidas en todo el mundo, permitiendo la comparación entre ellas desde el punto de vista nutricional. Además, se comprobó que las muestras cultivadas y silvestres de la misma especie tienen una composición química diferente, incluyendo azúcares solubles, ácidos grasos y perfil de tocoferoles.

Abstract

Herein, it was reported and compared the chemical composition and nutritional value of the most consumed species as fresh cultivated mushrooms: *Agaricus bisporus* (white and brown mushrooms), *Pleurotus ostreatus* (oyster mushroom), *Pleurotus eryngii* (King oyster mushroom), *Lentinula edodes* (shiitake) and *Flammulina velutipes* (Golden needle mushroom). Shiitake revealed the highest levels of macronutrients, unless proteins, as also the highest sugars, tocopherols and PUFA levels, and the lowest SFA content. White and brown mushrooms showed similar macronutrients composition, as also similar values of total sugars, MUFA, PUFA and total tocopherols. Oyster and king oyster mushrooms gave the highest MUFA contents with similar contents in PUFA, MUFA and SFA in both samples. They also revealed similar moisture, ash, carbohydrates and energy values. This study contributes to the elaboration of nutritional databases of the most consumed fungi species worldwide, allowing comparison between them. Moreover it was reported that cultivated and the wild samples of the same species have different chemical composition, including sugars, fatty acids and tocopherols profiles.

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Chemical composition and nutritional value of the most widely appreciated cultivated mushrooms: An inter-species comparative study

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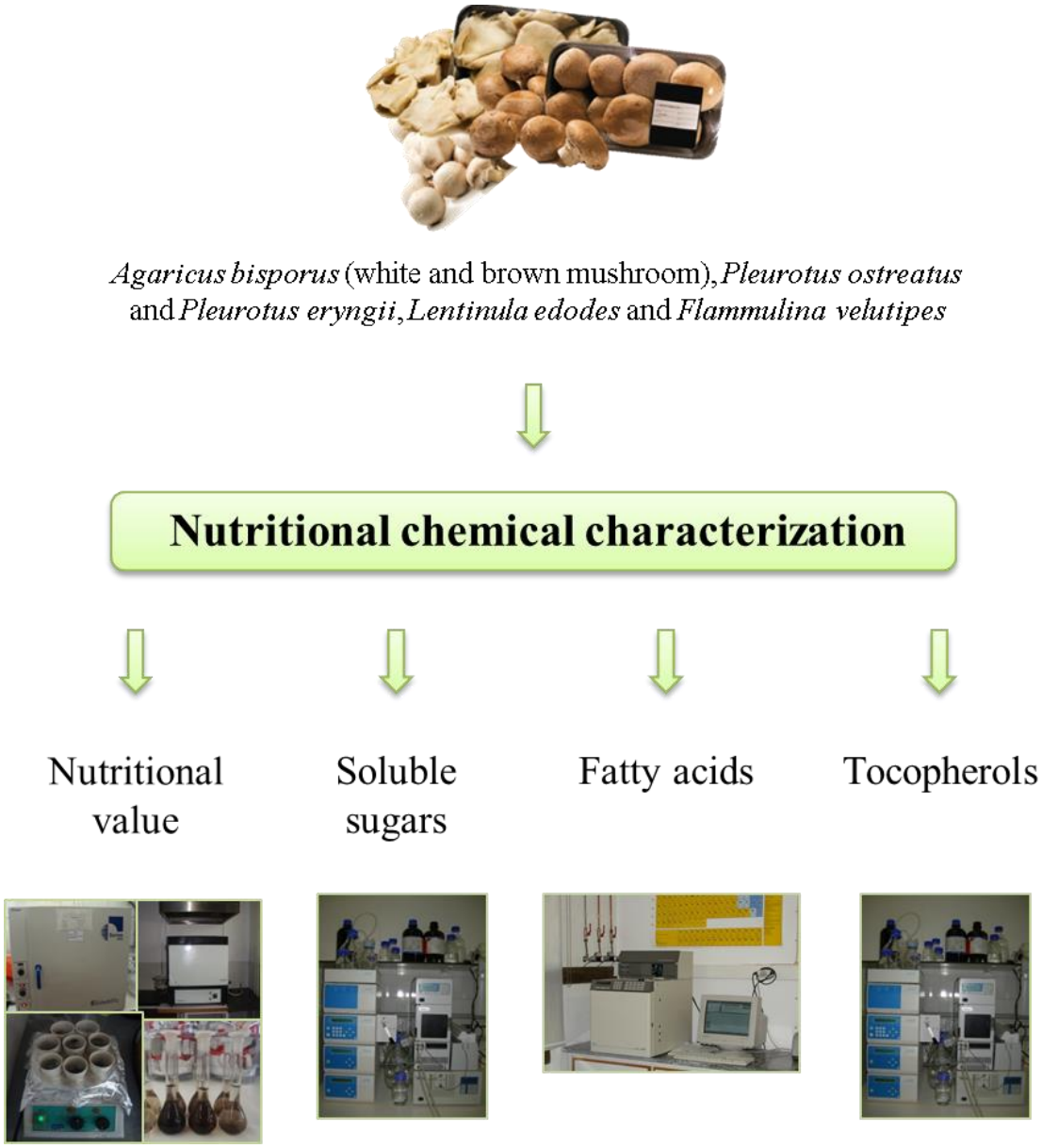

Figure 27. Graphical abstract article 1.

Article 2: Antioxidant properties and phenolic profile of the most widely appreciated cultivated mushrooms: A comparative study between *in vivo* and *in vitro* samples

Reference: Filipa S. Reis, Anabela Martins, Lillian Barros, Isabel C.F.R. Ferreira. (2012). *Food and Chemical Toxicology*, 50, 1201-1207.
DOI: 10.1016/j.fct.2012.02.013

Resumen

En el presente trabajo se aborda la caracterización y comparación de la capacidad antioxidantes y el perfil fenólico de algunas de las especies de setas cultivadas más consumidas y sus correspondientes micelios obtenidos mediante técnicas *in vitro*, como son: *Agaricus bisporus* (champiñón común y portobello), *Pleurotus ostreatus* (gírgola o champiñón ostra), *Pleurotus eryngii* (seta de cardo) y *Lentinula edodes* (shiitake). Para la determinación de la actividad antioxidante, se emplearon diferentes métodos analíticos, evaluando el poder reductor (ensayo de *Folin-Ciocalteu* y Ferricianide/Prussian blue), actividad antiradicalaria (ensayo 2,2-diphenil-1-picrilhydrazil (DPPH)) y la inhibición de la peroxidación lipídica (el ensayo de inhibición de la decoloración del β-caroteno y el ensayo de inhibición de especies reactivas del ácido tiobarbitúrico (TBARS)) de las muestras objeto d estudio. El análisis de compuestos fenólicos se realizó por HPLC/DAD. La especie estudiada con mayor potencial antioxidante fué *A. bispous* (portobello), mientras que el micelio obtenido a partir de *L. edodes* fue el que mayor actividad antioxidantes presentó frente al ensayo del poder reductor. Generalmente, las muestras obtenidas *in vivo* revelaron propiedades antioxidantes mayores que sus micelios obtenidos por técnicas *in vitro*. En relación a los compuestos fenólicos, se cuantificaron, tanto en setas y micelios, sin ninguna abundancia en particular. Los resultados mostraron que no existe correlación entre los hongos comerciales estudiados y sus micelios correspondientes, sin embargo, este estudio contribuye al aumento de los datos relativos a algunas de las especies consumidas en fresco y la posibilidad de su producción *in vitro* como fuente de compuestos bioactivos.

Abstract

The present study reports a comparison of the antioxidant properties and phenolic profile of the most consumed species as fresh cultivated mushrooms and their mycelia produced in vitro: *Agaricus bisporus* (white and brown), *Pleurotus ostreatus* (oyster), *Pleurotus eryngii* (king oyster) and *Lentinula edodes* (shiitake). The antioxidant activity was evaluated through reducing power (*Folin-Ciocalteu* and Ferricyanide/Prussian blue assays), free radical scavenging activity (DPPH assay) and lipid peroxidation inhibition (β-carotene/linoleate and TBARS assays). The analysis of phenolic compounds was performed by HPLC/PAD. The mushroom species with the highest antioxidant potential was *A. bispous* (brown). However, concerning to the species obtained *in vitro*, it was *L. edodes* that demonstrate the highest reducing power. Generally, *in vivo* samples revealed higher antioxidant properties than their mycelia obtained by *in vitro* techniques. About the phenolic compounds researched, they were detected both in mushrooms and mycelia without any particular abundance. Results showed that there is no correlation between the studied commercial mushrooms and the corresponding mycelia obtained *in vitro*. Nevertheless, this study contributes to the rise of data relatively to the species consumed as fresh mushrooms and the possibility of their in vitro production as a source of bioactive compounds.

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Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Antioxidant properties and phenolic profile of the most widely appreciated cultivated mushrooms: A comparative study between *in vivo* and *in vitro* samples

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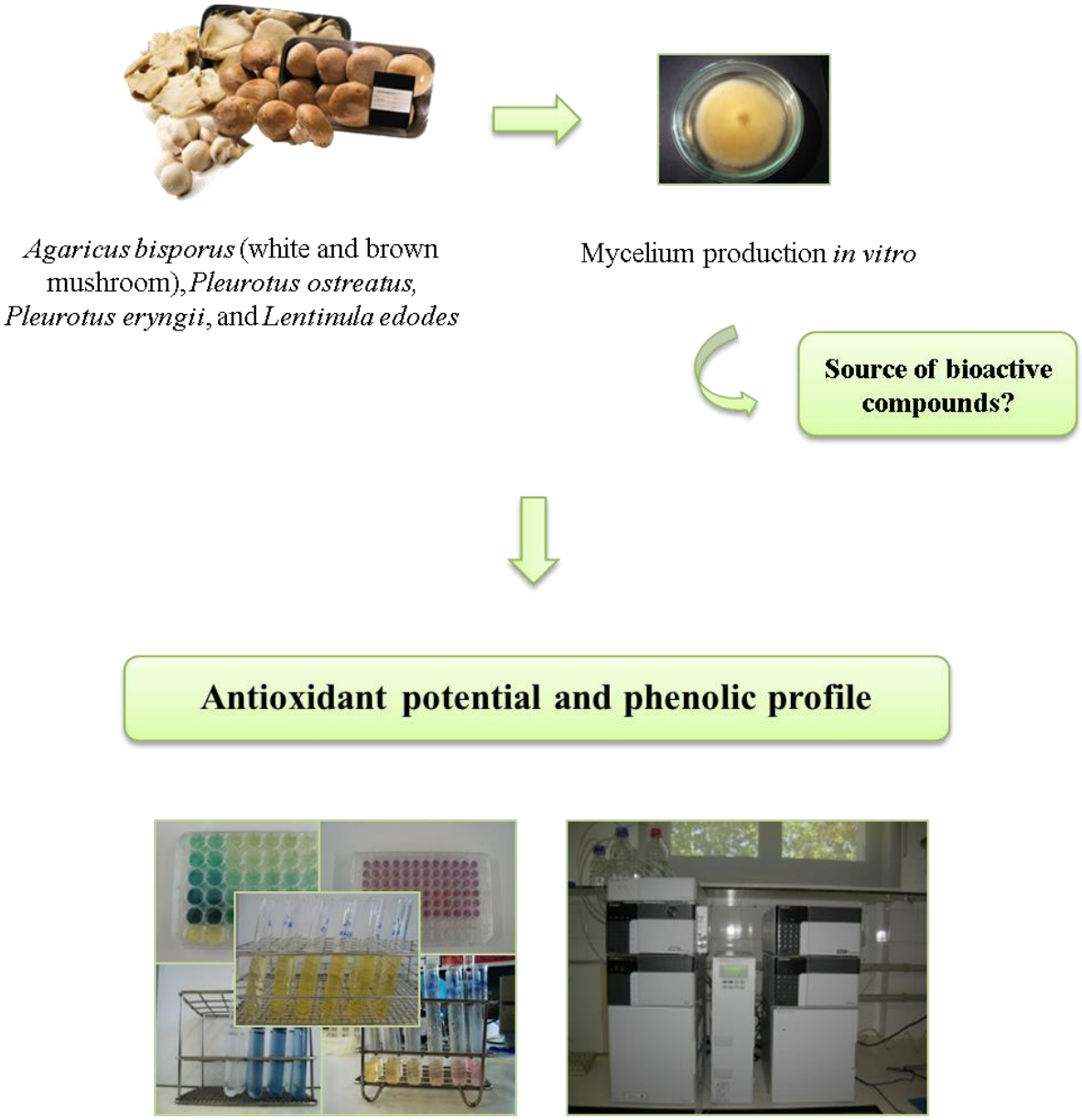

Figure 28. Graphical abstract article 2.

Article 3: Toward the antioxidant and chemical characterization of mycorrhizal mushrooms from Northeast Portugal

Reference: Filipa S. Reis, Sandrina A. Heleno, Lillian Barros, Maria João Sousa, Anabela Martins, Celestino Santos-Buelgas, Isabel C.F.R. Ferreira. (2011). *Journal of Food Science*, 76, 824-830.
DOI: 10.1111/j.1750-3841.2011.02251.x

Resumen

Las setas son muy apreciadas en todo el mundo por sus propiedades nutritivas y por su posible potencial farmacológico como fuentes de importantes compuestos bioactivos. Los hongos micorrizicos establecen asociaciones simbióticas con las raíces de las plantas, dichas simbiosis pueden influir en la producción de metabolitos secundarios, incluyendo los denominados compuestos bioactivos. El presente estudio se centra en la evaluación de la composición química y potencial antioxidante de algunas especies de setas micorrizicas del nordeste de Portugal: *Amanita caesarea, Cortinarius violaceus, Lactarius volemus* y *Suillus luteus*. De los resultados obtenidos, se observó un perfil similar de los compuestos analizados en las especies estudiadas con el siguiente orden: azúcares solubles > grasa > ácido ascórbico > compuestos fenólicos > tocoferoles. Sin embargo, se observó la prevalencia de azúcares solubles en *L. volemus*, compuestos fenólicos en *A. caesarea* y *C. violaceus*, y tocoferoles en *S. luteus*. Además, esta última se destacó por su potencial antioxidante.

Abstract

Mushrooms are widely appreciated all over the world for their nutritional properties and pharmacological value as sources of important bioactive compounds. Mycorrhizal macrofungi associate with plant roots constituting a symbiotic relationship. This symbiosis could influence the production of secondary metabolites, including bioactive compounds. We focused on the evaluation of antioxidant potential and chemical composition of mycorrhizal mushrooms species from Northeast Portugal: *Amanita caesarea, Cortinarius violaceus,*

Lactarius volemus, and *Suillus luteus*. A similar profile of metabolites was observed in the studied species with the order sugars > fat > ascorbic acid > phenolic compounds > tocopherols. Nevertheless, the samples revealed different compositions: prevalence of sugars in *L. volemus*, phenolic compounds in *A. caesarea* and *C. violaceus*, and tocopherols and antioxidant activity in *S. luteus*.

Journal of Food Science
A Publication of the Institute of Food Technologists

Toward the Antioxidant and Chemical Characterization of Mycorrhizal Mushrooms from Northeast Portugal

Filipa S. Reis, Sandrina A. Heleno, Lillian Barros, Maria João Sousa, Anabela Martins, Celestino Santos-Buelga, Isabel C.F.R. Ferreira

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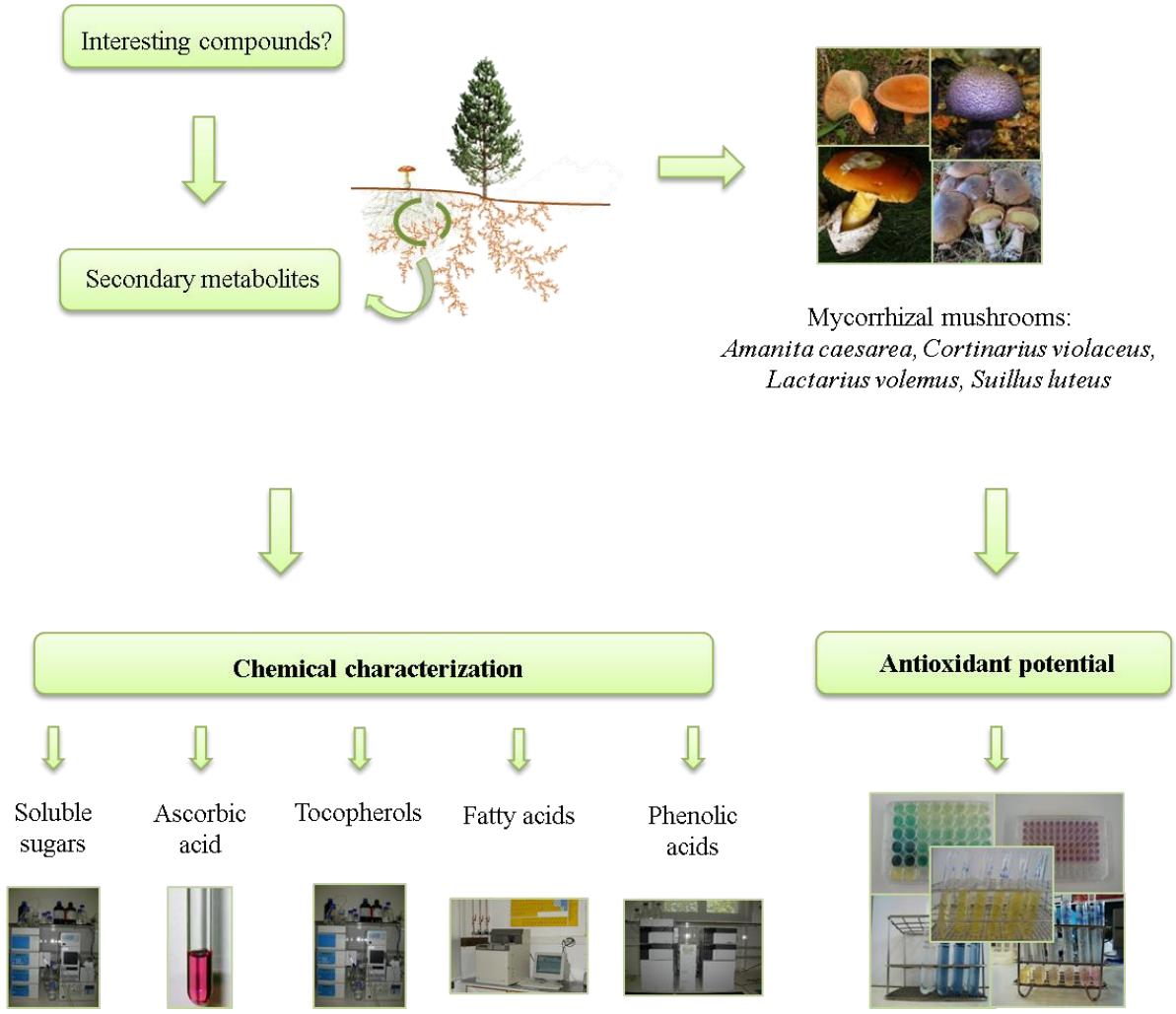

Figure 29. Graphical abstract article 3.

Article 4: Analytical methods applied to the chemical characterization and antioxidant properties of three wild edible mushroom species from Northeastern Portugal

Reference: Filipa S. Reis, Lillian Barros, Maria João Sousa, Anabela Martins, Isabel C.F.R. Ferreira. (2014). *Food Analytical Methods*, 7, 645-652.
DOI: 10.1007/s12161-013-9668-7

Resumen

En el presente estudio se ha comparado la composición química y el potencial antioxidante de tres especies de hongos silvestres procedentes del nordeste de Portugal, concretamente *Agaricus albertii*, *Agaricus urinascens* var. *excellens* y *Pleurotus eryngii*. Para la evaluación del valor nutricional, se siguieron procedimientos estándars validados (AOAC), mientras que se emplearon diferentes métodos cromatográficos para analizar azúcares solubles, ácidos grasos, tocoferoles, compuestos fenólicos y ácidos orgánicos. La actividad antioxidante se evaluó a través de los ensayos de poder reductor, actividad antiradicalaria y la inhibición de la peroxidación lipídica. *P. eryngii* destacó por su contenido en todos los macronutrientes, excepto en proteínas, asi como por su contenido de azúcares solubles, tocoferoles y ácidos grasos monoinsaturados. *A. albertii* y *A. urinascens* var. e*xcellens* presentaron una composición similar de macronutrientes. Sin embargo, en *A. albertii* destacó por su contenido de AGPI y compuestos fenólicos. *P. eryngii* presentó los mejores resultados de actividad antioxidante frente al poder reductor y actividad antiradicalaria, y *A. albertii* frente el ensayo de inhibición de la peroxidación lipídica.
Este estudio proporciona una caracterización química detallada, así como la evaluación del potencial antioxidante, de tres especies de setas silvestres procedentes de Portugal que todavía no habían sido estudiadas en detalle, contribuyendo al conocimiento y conservación de estos recursos como fuentes de compuestos de interés.

Abstract

The chemical composition and the antioxidant potential of three species of wild mushrooms from Northeastern Portugal, namely *Agaricus albertii*, *Agaricus urinascens* var. *excellens*, and *Pleurotus eryngii*, were compared. Standard procedures were followed in the nutritional value evaluation, while chromatographic procedures were used to analyze free sugars, fatty acids, tocopherols, phenolic compounds, and organic acids. To assess the antioxidant potential, reducing power, radical-scavenging activity, and lipid peroxidation inhibition were evaluated. *P. eryngii* revealed the highest levels of macronutrients, except proteins, as also the highest sugars, tocopherols, and monounsaturated fatty acids contents. *A. albertii* and *A. urinascens* var. *excellens* showed similar macronutrients composition. However, *A. albertii* revealed the highest content in PUFA and phenolic compounds. *P. eryngii* revealed the highest reducing power and radical-scavenging activity and *A. albertii* the highest lipid peroxidation inhibition.

This study provides a detailed chemical characterization and antioxidant potential evaluation of three species of wild mushrooms from Portugal not yet previously reported. Thus, this work intended to contribute to the increase of information concerning species of edible mushrooms (directed to the scientific community and general population) as well as contribute to the conservation of these resources as sources of compounds of interest.

Food Analytical Methods
March 2014, Volume 7, Issue 3, pp 645-652

Analytical Methods Applied to the Chemical Characterization and Antioxidant Properties of Three Wild Edible Mushroom Species from Northeastern Portugal

Authors

Filipa S. Reis, Lillian Barros, Maria João Sousa, Anabela Martins, Isabel C. F. R. Ferreira

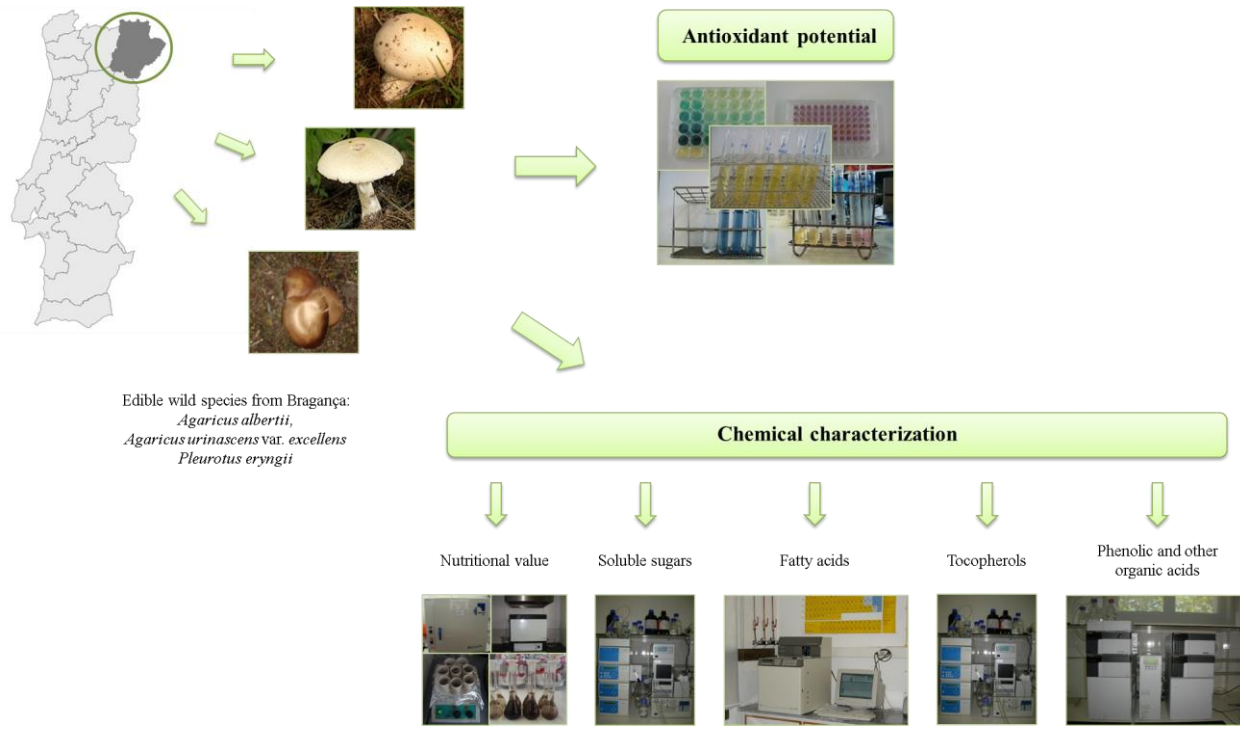

Figure 30. Graphical abstract article 4.

Article 5: Can *Suillus granulatus* (L.) Roussel be classified as a functional food?

Reference: Filipa S. Reis, Dejan Stojković, Lillian Barros, Jasmina Glamočlija, Ana Ćirić, Marina Soković, Anabela Martins, M. Helena Vasconcelos, Patricia Morales, Isabel C. F. R. Ferreira. (2014). *Food & Function*, 5, 2861-2869.
DOI: 10.1039/C4FO00619D

Resumen

El presente trabajo describe una caracterización química detallada de *Suillus granulatus*, además de evaluar las propiedades antioxidantes y antimicrobianas de sus extractos metanólicos. El estudio se realizó con muestras recolectadas en Portugal y Serbia para demostrar que, aunque las setas están fuertemente influenciadas por el medio en el que se desarrollan, tienen un perfil químico específico que puede ser típico de su género/especie. Ambas muestras demonstraron ser alimentos saludables, bajos en grasa y ricos en proteínas e hidratos de carbono, siendo el manitol y la trehalosa los principales azúcares solubles detectados. También demostraron ser fuente de ácidos orgánicos y compuesos fenólicos, así como ácidos grasos mono y poliinsaturados y tocoferoles. Las muestras procedentes de Serbia revelaron un mayor potencial antioxidante y antimicrobiano, en comparación con las muestras recolectadas en Portugal. En consecuencia, podríamos afirmar que *S. granulatus* puede considerarse un alimento funcional, ya que es una fuente de compuestos funcionales y biológicamente activos.

Abstract

The present work outlines a detailed chemical characterization of *Suillus granulatus* species, besides presenting the antioxidant and antimicrobial properties of their methanolic extracts. The study was carried out with samples drawn from Portugal and Serbia in order to prove that though mushrooms are strongly influenced by the environment in which they develop, they have a specific chemical profile that can be typical of their genus/species. The studied species

proved to be healthy foods, low in fat and rich in protein and carbohydrates, with mannitol and trehalose being the main free sugars detected. They also proved to be a source of organic and phenolic acids, as well as mono- and polyunsaturated fatty acids and tocopherols. The Serbian samples revealed higher antioxidant and antimicrobial potential. Accordingly, we find that the *S. granulatus* species can be considered to be a functional food, since it is a source of nutraceutical and biologically active compounds.

Food & Function

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Cite this: *Food Funct.*, 2014, **5**, 2861

Can *Suillus granulatus* (L.) Roussel be classified as a functional food?

Filipa S. Reis,[a,b,c] Dejan Stojković,[d] Lillian Barros,[a] Jasmina Glamočlija,[d] Ana Ćirić,[d] Marina Soković,[d] Anabela Martins,[a] M. Helena Vasconcelos,[c,e] Patricia Morales[b] and Isabel C. F. R. Ferreira*[a]

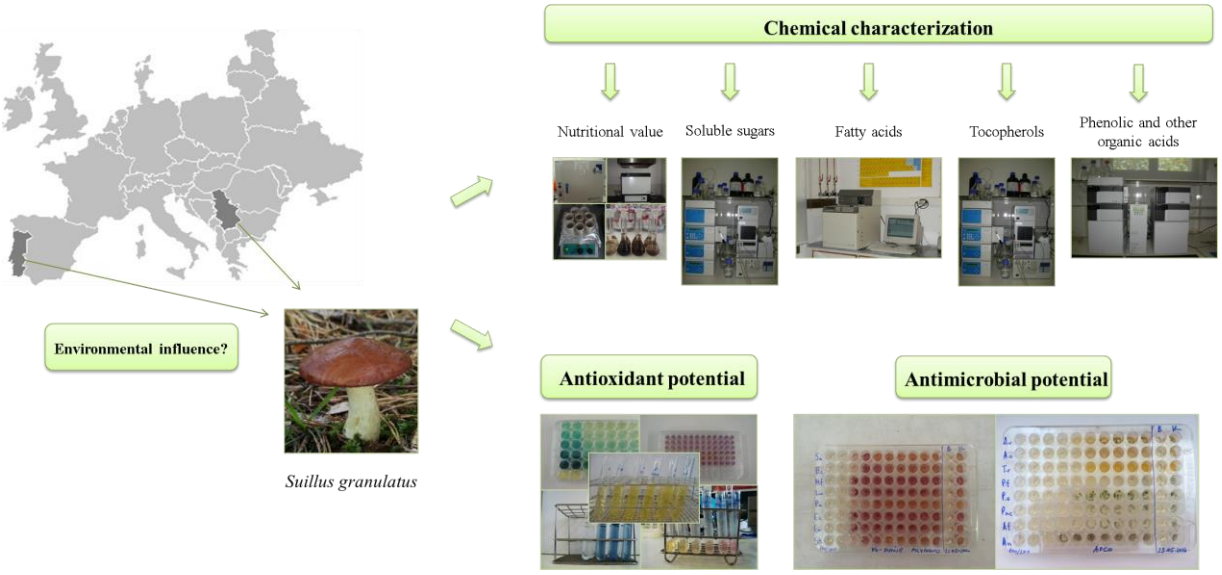

Figure 31. Graphical abstract article 5.

Article 6: The methanolic extract of *Cordyceps militaris* (L.) Link fruiting body shows antioxidant, antibacterial, antifungal and antihuman tumor cell lines properties

Reference: Filipa S. Reis, Lillian Barros, Ricardo C. Calhelha, Ana Ćirić, Leo J.I.D. van Griensven, Marina Soković, Isabel C.F.R. Ferreira. (2013). *Food and Chemical Toxicology*, 62, 91-98.
DOI: 10.1016/j.fct.2013.08.033

Resumen

Como *Cordyceps militaris* (L.) Link se conoce como una seta comestible con propiedades medicinales, de modo que en este trabajo se pretende caracterizar algunos compuestos bioactivos interesantes que podrían aislarse de esta especie y justificar esas propiedades biológicas. Los compuestos hidrófilos y lipófilos se analizaron al través de técnicas cromatográficas acopladas a diferentes detectores. El extracto metanólico de *C. militaris* se empleó para evualuar las posibles propiedades antioxidantes, antibacterianas, antifúngicas y antiproliferativas en diferentes líneas de células tumorales humanas. El mannitol (2,01 g/100 g) y trehalosa (24,71 g/100 g) fueron los azúcares solubles mayoritarios en *C. militaris*, así como se detectaron los ácidos oxálico, cítrico y fumárico (0,33, 7,97 y 0,13 g/100 g, respectivamente). El ácido *p*-hidroxibenzoico fue el único ácido fenólico cuantificado en esta especie (0,02 mg/100 g); aunque también se encontró ácido cinámico (0,11 mg/100 g). Los ácidos grasos poliinsaturados (68,87%) predominaron sobre los ácidos grasos saturados (23,40%) y el δ-tocoferol fue la única isoforma de la vitamina E detectada (55,86 μg/100 g). El extracto metanólico de *C. militaris* presentó buenos resultados frente al ensayo de inhibición la peroxidación lipídica, poder reductor y actividad antiradicalaria. Este extracto también demostró tener actividad antibacterianas y antifúngicas, así como la capacidad de inhibir la proliferación de líneas celulares de carcinoma humano MCF-7 (mama), NCI-H460 (pulmón), HCT-15 (colon) y HeLa (cervical).

Abstract

Being *Cordyceps militaris* (L.) Link recognized as a medicinal and edible mushroom, this work intends to reveal new interesting bioactive molecules that could be isolated from this species. Hydrophilic and lipophilic compounds were analyzed by chromatographic techniques coupled to different detectors. The methanolic extract of *C. militaris* was tested for its antioxidant, antibacterial, antifungal and antiproliferative properties in different human tumor cell lines. Mannitol (2.01 g/100 g dw) and trehalose (24.71 g/100 g) were the free sugars found in *C. militaris*. Polyunsaturated fatty acids (68.87%) predominated over saturated fatty acids (23.40%) and d-tocopherol was the only isoform of vitamin E detected (55.86 lg/100 g). The organic acids found in this mushroom were oxalic, citric and fumaric acids (0.33, 7.97 and 0.13 g/100 g, respectively). *p*-Hydroxybenzoic acid was the only phenolic acid quantified in this species (0.02 mg/100 g); although cinnamic acid was also found (0.11 mg/100 g). The methanolic extract of *C. militaris* proved to inhibit lipid peroxidation, have reducing power and scavenge free radicals. This extract also revealed strong antibacterial and antifungal properties. Finally, the *C. militaris* extract was able to inhibit the proliferation of MCF-7 (breast), NCI-H460 (non-small lung), HCT-15 (colon) and HeLa (cervical) human carcinoma cell lines.

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

The methanolic extract of *Cordyceps militaris* (L.) Link fruiting body shows antioxidant, antibacterial, antifungal and antihuman tumor cell lines properties

Filipa S. Reis[a], Lillian Barros[a], Ricardo C. Calhelha[a], Ana Ćirić[b], Leo J.L.D. van Griensven[c], Marina Soković[b,*], Isabel C.F.R. Ferreira[a,*]

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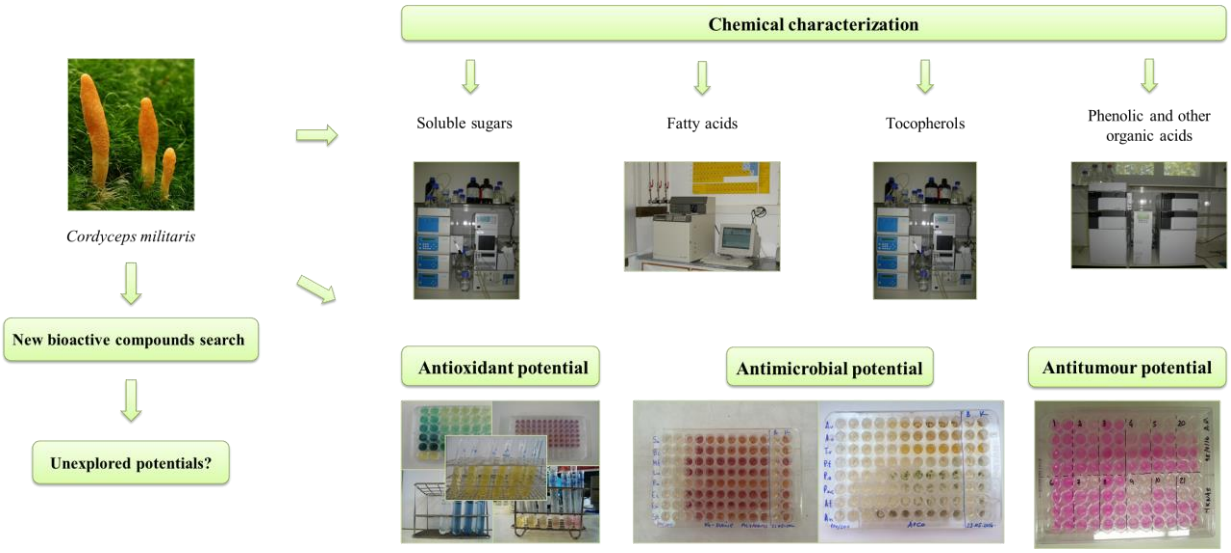

Figure 32. Graphical abstract article 6.

Article 7: Chemical characterization of the medicinal mushroom *Phellinus linteus* (Berkeley & Curtis) Teng and contribution of different fractions to its bioactivity

Reference: Filipa S. Reis, João C.M. Barreira, Ricardo C. Calhelha, Leo J.L.D. van Griensven, Ana Ćirić, Jasmina Glamočlija, Marina Soković, Isabel C.F.R. Ferreira. (2014). *LWT – Food Science and Technology*, 58, 478-485.
DOI: 10.1016/j.lwt.2014.04.013

Resumen

Las setas son ampliamente apreciadas por sus propiedades organolépticas, siendo también reconocidas como una buena fuente de compuestos bioactivos que proporcionan actividad antioxidante, antimicrobiana y citotóxica. Sus polisacáridos (incluyendo glucanos) se señalan con frecuencia como los compuestos más bioactivos aislados de setas, pero otros compuestos tales como triterpenoides, también podrían destacarse por su bioactividad. La bioactividad de *Phellinus linteus* se evaluó en las fracciones seleccionadas (polisacáridos, glucanos y triterpenoides), así como en el extracto metanólico y etanólico, dado que en muchas ocasiones, cuando se analizan compuestos aislados, los posibles efectos sinérgicos se pueden perder. Los mejores resultados obtenidos frente a la actividades antioxidante y antibacteriana se observaron en el extracto metanolico, mientras que las fracciones de glucano y triterpenoides mostrarón la mayor actividad antifúngica. Por el contrario, lo extracto etanólico reveló los mejores resultados frente a la actividad citotóxica. Los resultados obtenidos en este estudio ponen de manifiesto que de los extractos de *P. linteus* y sus fracciones podrían ser útiles para encontrar compuestos antimicrobianos, antioxidantes y agentes citotóxicos, como alternativas a las sustancias sintéticas empleadas en la agricultura, la industria alimentaria y/o la industria farmacéutica.

Abstract

Mushrooms are widely appreciated for their organoleptic qualities, being also recognized as good sources of bioactive compounds that provide antioxidant, antimicrobial and cytotoxic activities. Polysaccharides (including glucans) are often pointed out as the most bioactive compounds isolated from mushrooms, but other molecules such as triterpenoids, might also be highlighted for their bioactivity. In scientific research, when isolated compounds are used, potential synergistic effects might be lost. Accordingly, the bioactivity of *Phellinus linteus* was evaluated in selected fractions (polysaccharides, glucans and triterpenoids), as well as in the methanolic and ethanolic extracts. The best antioxidant and antibacterial activities were obtained with methanolic extract, while glucan and triterpenoid fractions gave the strongest antifungal activity. In contrast, ethanolic extract gave the best results in cytotoxic activity, indicating that the bioactive compounds present might act synergistically. The differentiated activity of *P. linteus* fractions and extracts could be useful to find antimicrobial, antioxidant and cytotoxic agents as alternatives to synthetic chemicals with application in agriculture, food industry and/or pharmacy.

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Chemical characterization of the medicinal mushroom *Phellinus linteus* (Berkeley & Curtis) Teng and contribution of different fractions to its bioactivity

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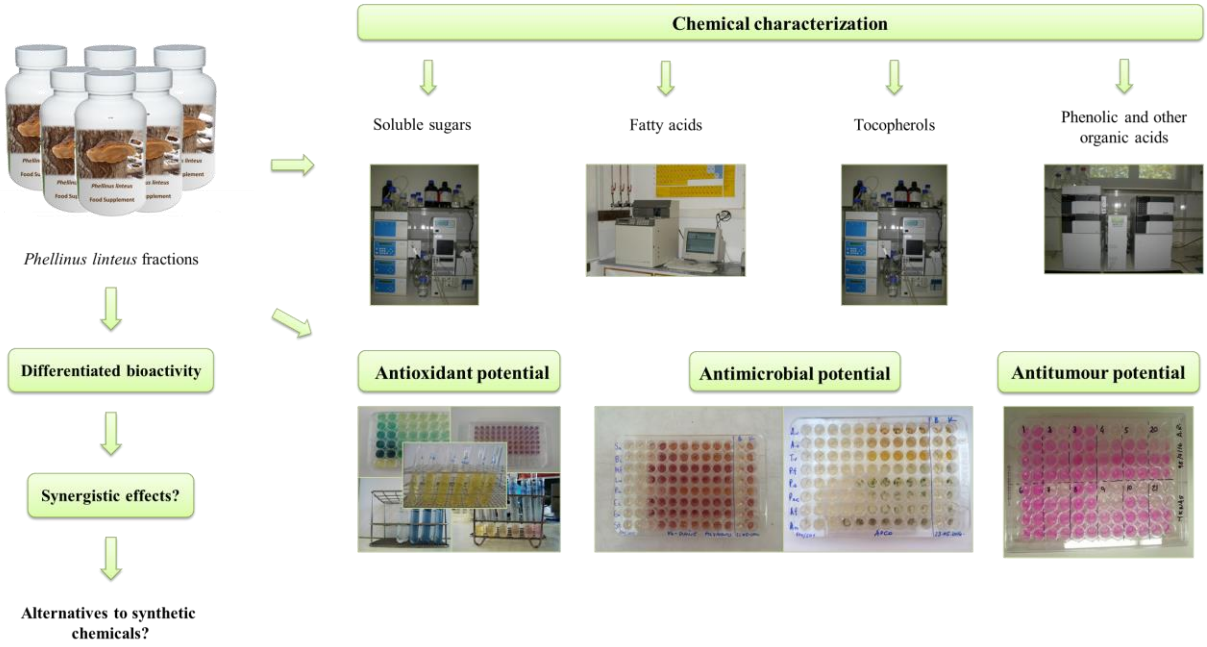

Figure 33. Graphical abstract article 7.

Article 8: *Leccinum molle* (Bon) Bon and *Leccinum vulpinum* Watling: The first study of their nutritional and antioxidant potential

Reference: Filipa S. Reis, Lillian Barros, Anabela Martins, M. Helena Vasconcelos, Patricia Morales, Isabel C.F.R. Ferreira. (2016). *Molecules*, 21, 246.
DOI: 10.3390/molecules21020246

Resumen

En este trabajo se presenta el perfil químico de dos especies comestibles de setas del género *Leccinum*: *Leccinum molle* (Bon) Bon y *Leccinum vulpinum* Watling, recolectados en las cercanías de Bragança (Noreste de Portugal). Ambas especies se caracterizaron con respecto a su contenido en nutrientes (azúcares solubles, ácidos grasos y vitaminas), no-nutrientes (ácidos fenólicos y otros ácidos orgánicos) y actividad antioxidante. Hasta donde sabemos, no se han realizado estudios previos sobre la caracterización química y la bioactividad de estas especies. En consecuencia, este estudio pretende aumentar la información disponible sobre las especies de hongos comestibles, así como resaltar otro factor importante en la conservación de los recursos micológicos: su potencial como fuentes de compuestos bioactivos. En general, ambas especies revelaron perfiles de nutrientes similares, con bajos niveles de grasa; fructosa, manitol y trehalosa como los principales azúcares solubles; así como elevados porcentajes de ácidos grasos monoinsaturados y poliinsaturados. Las especies estudiadas destacaron por la presencia de compuestos bioactivos, como ácidos fenólicos (ácido gálico, ácido protocatechúico y ácido *p*-hidroxibenzoico), ácidos orgánicos (ácidos cítrico y fumárico) y presentaron buenos resultados frente a los diferentes ensayos de actividad antioxidante.

Abstract

This work presents the chemical profile of two edible species of mushrooms from the genus *Leccinum*: *Leccinum molle* (Bon) Bon and *Leccinum vulpinum* Watling, both harvested on the outskirts of Bragança (Northeastern Portugal). Both species were prepared and characterized regarding their content in nutrients (*i.e.*, free sugars, fatty acids and vitamins), non-nutrients

(*i.e.*, phenolic and other organic acids) and antioxidant activity. To the best of our knowledge, no previous studies on the chemical characterization and bioactivity of these species have been undertaken. Accordingly, this study intends to increase the available information concerning edible mushroom species, as well as to highlight another important factor regarding the conservation of the mycological resources – their potential as sources of nutraceutical/pharmaceutical compounds. Overall, both species revealed similar nutrient profiles, with low fat levels, fructose, mannitol and trehalose as the foremost free sugars, and high percentages of mono- and polyunsaturated fatty acids. They also revealed the presence of bioactive compounds, namely phenolic (*e.g.*, gallic acid, protocatechuic acid and *p*-hydroxybenzoic acid) and organic acids (*e.g.*, citric and fumaric acids) and presented antioxidant properties.

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Article

Leccinum molle (Bon) Bon and *Leccinum vulpinum* Watling: The First Study of Their Nutritional and Antioxidant Potential

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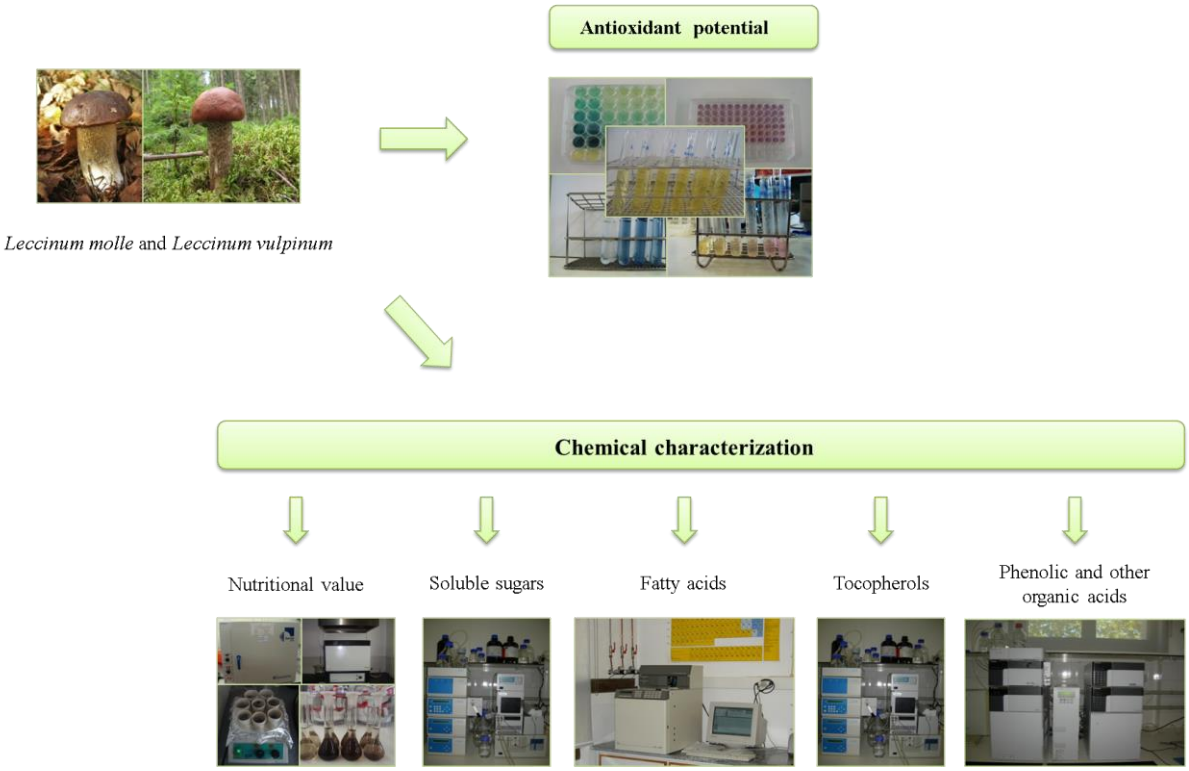

Figure 34. Graphical abstract article 8.

Article 9: *Leccinum vulpinum* Watling induces DNA damage, decreases cell proliferation and induces apoptosis on the human MCF-7 breast cancer cell line

Reference: Filipa S. Reis, Diana Sousa, Lillian Barros, Anabela Martins, Patricia Morales, Isabel C.F.R. Ferreira, M. Helena Vasconcelos. (2016). *Food and Chemical Toxicology*, 90, 45-54.
DOI: 10.1016/j.fct.2016.02.005

Resumen

El objetivo del presente trabajo fue estudiar la actividad antitumoral de un extracto fenólico (rico en ácidos hidroxibenzoicos) de la seta comestible *Leccinum vulpinum* Watling. En una primera aproximación, se ensayó el extracto frente a una serie de cuatro lineas celulares tumorales humanas. Posteriormente, debido a los buenos resultados obtenidos y a la evidencia científica existente en relación al consumo de esta seta y los datos de prevalencia del cancer de mama, se realizó un estudio detallado de la bioactividad del extracto seleccionado en células MCF-7, empleandose una línea celular de adenocarcinoma de mama y otra linea celular no maligna de mama MCF-10A (como control). En general, se observó una disminución de la proliferación celular y de la inducción de la apoptosis, además, los resultados obtenidos sugieren que el extracto fenólico podría causar daño en el ADN celular de las células MCF-7. De modo que, los resultados obtenidos ponen de manifiesto el potencial de esta seta como fuente de compuestos biológicamente activos, particularmente con actividad antitumoral.

Abstract

The current work aimed to study the antitumour activity of a phenolic extract of the edible mushroom *Leccinum vulpinum* Watling, rich essentially in hydroxybenzoic acids. In a first approach, the mushroom extract was tested against cancer cell growth by using four human tumour cell lines. Given the positive results obtained in these initial screening experiments

and the evidence of some studies for an inverse relationship between mushroom consumption and breast cancer risk, a detailed study of the bioactivity of the extract was carried out on MCF-7 cells. Once the selected cell line to precede the work was the breast adenocarcinoma cell line, the human breast non-malignant cell line MCF-10A was used as control. Overall, the extract decreased cellular proliferation and induced apoptosis. Furthermore, the results also suggest that the extract causes cellular DNA damage. Data obtained highlight the potential of mushrooms as a source of biologically active compounds, particularly with antitumour activity.

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Leccinum vulpinum Watling induces DNA damage, decreases cell proliferation and induces apoptosis on the human MCF-7 breast cancer cell line

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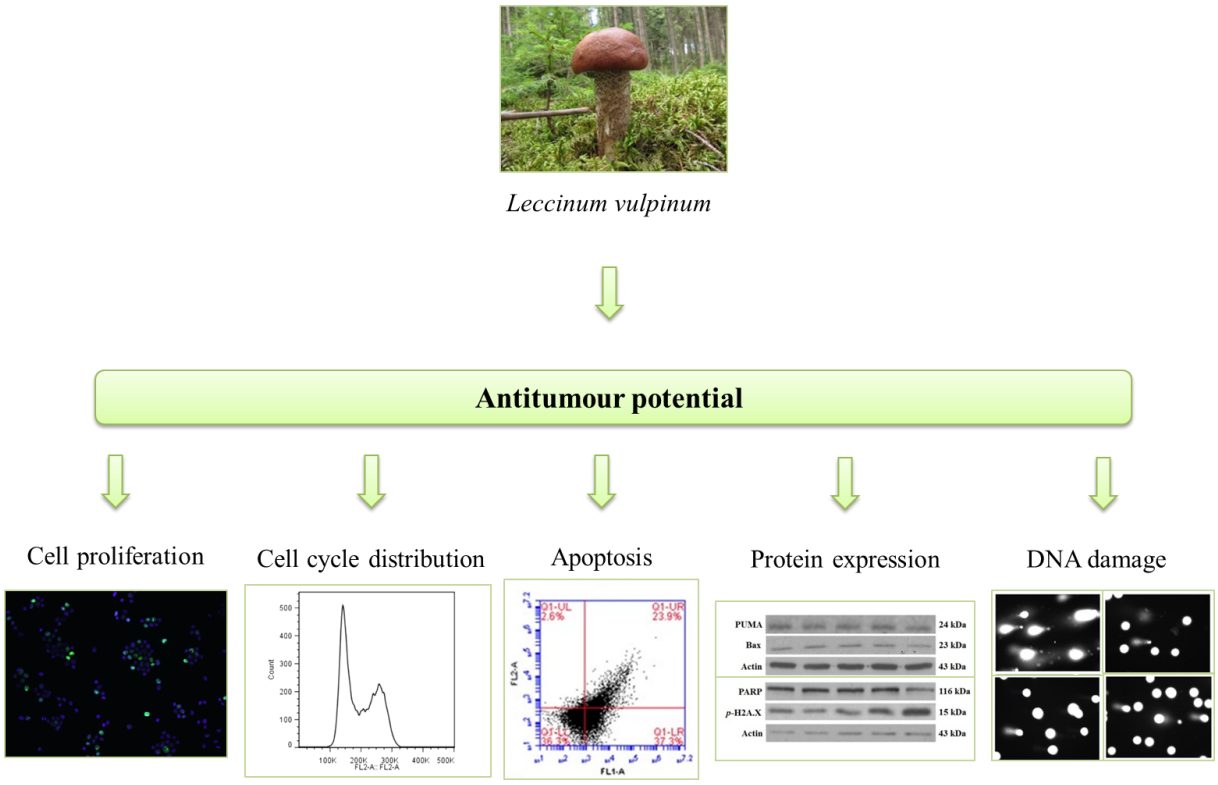

Figure 35. Graphical abstract article 9.

Article 10: Methanolic extract of *Ganoderma lucidum* induces autophagy of AGS human gastric tumor cells

Reference: Filipa S. Reis, Raquel T. Lima, Patricia Morales, Isabel C.F.R. Ferreira, M. Helena Vasconcelos. (2015). *Molecules*, 20, 17872-17882.
DOI: 10.3390/molecules201017872

Resumen

Ganoderma lucidum es una de las especies de setas más estudiadas hasta la fecha, particularmente en cuanto a sus propiedades medicinales. Estudios previos (incluyendo los llevados a cabo por los autores) han demostrado evidencias científicas de que el extracto metanólico de *G. lucidum* afecta a la autofagia celular. Sin embargo, no se sabe si induce autofagia o disminuye el flujo autofágico. Tras el tratamiento de una línea celular de adenocarcinoma gástrico (AGS) con el extracto metabólico obtenido a partir de esta seta, se observó un aumento de la formación de autofagosomas (vacuolas típicas de la autofagia), un aumento de los niveles celulares de LC3-II y una disminución de los niveles celulares de p62, lo que confirma que el extracto metabólico estudiado afecta a la autofagia celular. Así como, el tratamento de las células AGS con el extracto junto con inhibidores de proteasas lisosómicos, aumentaron los niveles celulares de LC3-II y p62. Los resultados obtenidos demostraron que, en las células AGS, el extracto metanólico de *G. lucidum* causa una inducción de autofagia, en lugar de una reducción en el flujo autofágico, elucidandose así uno de los posibles mecanismos de acción de dicho extracto frente a esta linea celular tumoral.

Abstract

Ganoderma lucidum is one of the most widely studied mushroom species, particularly in what concerns its medicinal properties. Previous studies (including those from some of us) have shown some evidence that the methanolic extract of *G. lucidum* affects cellular autophagy. However, it was not known if it induces autophagy or decreases the autophagic flux. The treatment of a gastric adenocarcinoma cell line (AGS) with the mushroom extract increased

the formation of autophagosomes (vacuoles typical from autophagy). Moreover, the cellular levels of LC3-II were also increased, and the cellular levels of p62 decreased, confirming that the extract affects cellular autophagy. Treating the cells with the extract together with lysossomal protease inhibitors, the cellular levels of LC3-II and p62 increased.

The results obtained proved that, in AGS cells, the methanolic extract of *G. lucidum* causes an induction of autophagy, rather than a reduction in the autophagic flux. To our knowledge, this is the first study proving that statement.

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Article

Methanolic Extract of *Ganoderma lucidum* Induces Autophagy of AGS Human Gastric Tumor Cells

Filipa S. Reis [1,2,3,4], Raquel T. Lima [1,2,5], Patricia Morales [3], Isabel C. F. R. Ferreira [4,*] and M. Helena Vasconcelos [1,2,6,*]

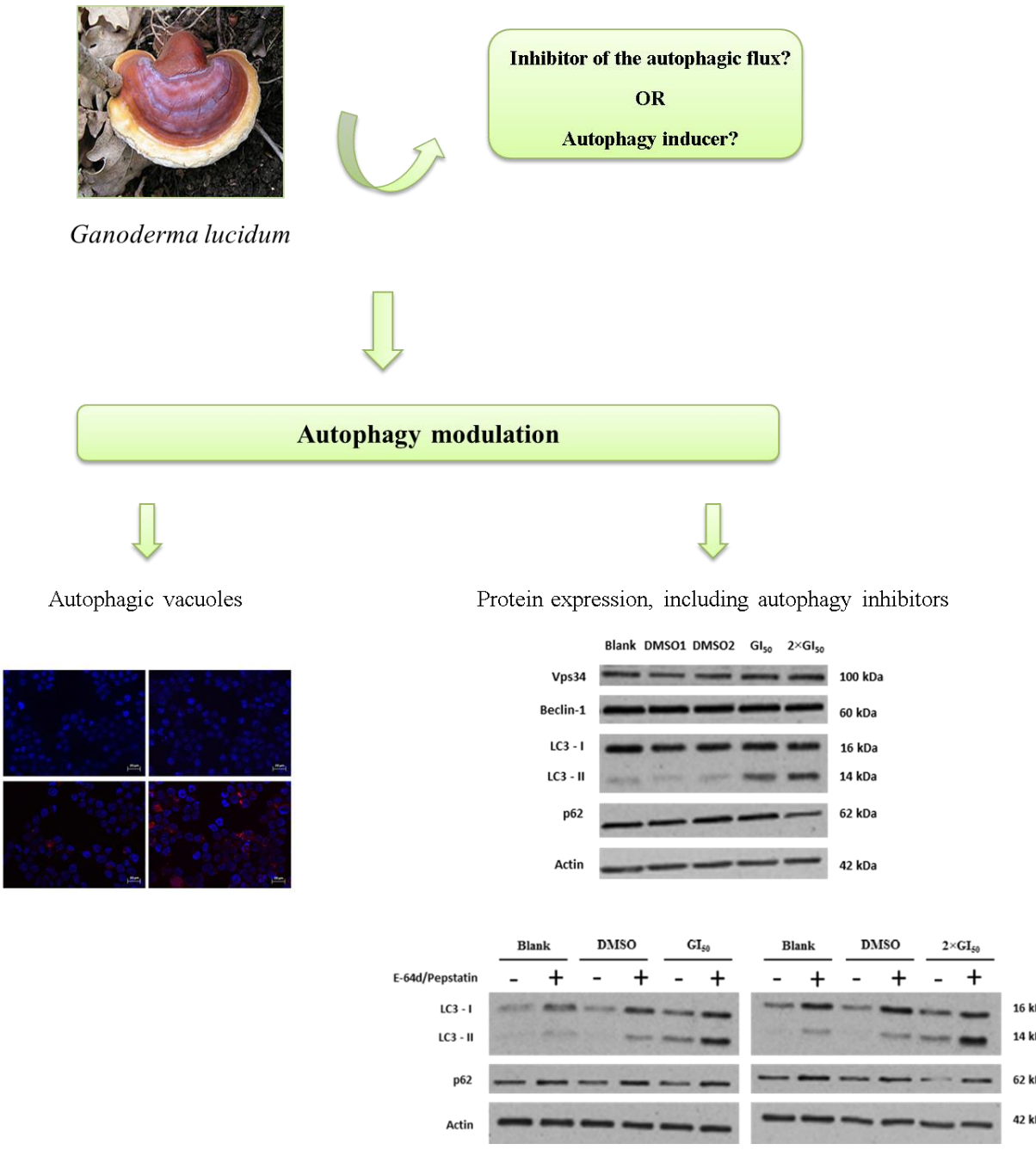

Figure 36. Graphical abstract article 10.

Part VI

Integrative Discussion

As previously referred, the present work started with the analysis of some commercially available mushroom species. The mushrooms selected were the most widely appreciated mushrooms (*Agaricus bisporus* (white and brown mushrooms), *Flammulina velutipes*, *Lentinula edodes*, *Pleurotus eryngii* and *Pleurotus ostreatus*). In addition, an inter-species comparative study was performed. Furthermore, some of these species were cultured by *in vitro* techniques to try to obtain greater concentrations of bioactive compounds (*e.g.*, phenolic and related compounds).

The remaining studied species comprised wild edible mushrooms from the Northeastern region of Bragança (Portugal). Moreover, three wild species from other countries were also considered, with the purpose of making some comparative studies (*Suillus granulatus*) which allowed the opportunity to study some medicinal species usually not consumed or used in the Portuguese culture (*Cordyceps militaris* and *Phellinus linteus*).

6.1. Mushrooms nutritional value

Regarding the nutritional value of the studied cultivated and wild mushrooms, in general, similar results were obtained (**Table 4**).

Carbohydrates were the main macronutrient present in all the species, and the highest levels were found in the cultivated *F. velutipes*, *L. edodes* and *P. ostreatus* (85.9 and 87.84 g/100 g dw) and mostly in *P. linteus* (95.3 g/100 g dw), which agrees with literature that states that carbohydrates constitute about one-half of mushroom dry matter (Kalač, 2013). Carbohydrates content includes also dietary fibre, such as the structural polysaccharides β-glucans, chitin, hemicelluloses and pectin substances (Kalač, 2009 and 2013). Since *P. linteus* is a thick, hard and woody mushroom, this could explain the particularly high carbohydrates content.

Table 4. Nutritional values of the studied edible mushroom species (mean ± SD).

Species	Total carbohydrates (g/100g dw)	Crude protein (g/100g dw)	Fat (g/100g dw)	Ash (g/100g dw)	Energy (kcal/100g dw)
Cultivated					
Agaricus bisporus (J.E. Lange) Imbach (white mushroom)	74.0 ± 0.9^{g}	14.1 ± 0.2^{d}	2.2 ± 0.2^{e}	10 ± 1^{f}	372 ± 3^{d}
Agaricus bisporus (J.E. Lange) Imbach (brown mushroom)	71.1 ± 0.3^{fg}	15.7 ± 0.1^{b}	1.7 ± 0.1^{f}	11.4 ± 0.3^{e}	362.92 ± 0.06^{e}
Pleurotus ostreatus (Jacq. Ex Fr.) P. Kumm.	85.9 ± 0.3^{e}	7.0 ± 0.3^{g}	1.42 ± 0.08^{g}	5.7 ± 0.4^{i}	384 ± 2^{bc}
Pleurotus eryngii (DC.) Quél.	$74 \pm 5f^{gh}$	11.0 ± 0.3^{f}	1.5 ± 0.1^{g}	6.2 ± 0.5^{hi}	420 ± 24^{ab}
Lentinula edodes (Berk.) Pegler	87.1 ± 0.3^{d}	4.4 ± 0.4^{j}	1.75 ± 0.08^{f}	6.7 ± 0.5^{h}	381.9 ± 0.2^{cd}
Flammulina velutipes (Curtis) Singer	87.84 ± 0.08^{c}	3.89 ± 0.08^{j}	1.8 ± 0.1^{f}	7.2 ± 0.8^{gh}	376 ± 5^{d}
Phellinus linteus (Berkeley & Curtis) Teng	95.3 ± 0.2^{a}	2.8 ± 0.3^{k}	0.49 ± 0.03^{h}	1.33 ± 0.05^{j}	397.14 ± 0.05^{a}
Wild					
Agaricus albertii (Bon.)	56.7 ± 0.3^{i}	19.83 ± 0.02^{a}	1.38 ± 0.03^{g}	22.1 ± 0.3^{b}	318 ± 1^{g}
Agaricus urinascens var. *excellens* (F.H. Møller) Nauta	55 ± 1^{i}	14.5 ± 0.4^{cd}	1.4 ± 0.1^{g}	29.6 ± 0.8^{a}	288 ± 3^{h}
Pleurotus eryngii (DC.) Quél.	78.6 ± 0.5^{fg}	2.090 ± 0.006^{j}	4.36 ± 0.06^{a}	15.0 ± 0.4^{c}	362 ± 1^{e}
Suillus granulatus (L.) Roussel (Portugal)	73.5 ± 0.3^{h}	14.8 ± 0.3^{c}	3.74 ± 0.09^{b}	8.0 ± 0.4^{g}	387 ± 2^{abc}
Suillus granulatus (L.) Roussel (Serbia)	89.353 ± 0.005^{b}	7.929 ± 0.001^{l}	0.27 ± 0.04^{i}	10.38 ± 0.04^{f}	359.8 ± 0.4^{f}
Leccinum molle Bon (Bon)	$78.4 \pm 0.6f^{h}$	13.1 ± 0.6^{e}	2.80 ± 0.07^{d}	5.7 ± 0.3^{i}	391.2 ± 0.6^{a}
Leccinum vulpinum Watling	72.9 ± 0.3^{h}	10.5 ± 0.3^{f}	2.97 ± 0.06^{c}	13.6 ± 0.4^{d}	360 ± 2^{e}

The different accuracy in the expression of the results is explained by the maintenance of the decimal places allowed by the magnitude of standard deviation. In each column, different letters mean statistical significant differences among the samples. Significance level was set as $p<0.05$.

Mushrooms are reported to be a good source of protein, being the amino acid composition comparable with protein of animal origin. Indeed, it has been reported that leucine, valine, glutamine, glutamic and aspartic acids are the most abundant amino acids in mushrooms (Asgar et al., 2010; Léon-Guzmán et al., 1997; Longvah & Deosthale, 1998; Mattila et al., 2002; Mdachi et al., 2004; Ouzouni et al., 2009; Ribeiro et al., 2008a). The crude protein content calculated for the studied species varies between 2.090 and 19.83 g/100 g dw. Although some lower values were obtained especially for the cultivated mushrooms *F. velutipes*, *L. edodes* and *P. ostreatus,* and for the wild species *P. eryngii*, the values obtained for the remaining samples are close to the range of values reported in the literature (22.8 and 24.9 g/100g dw; Kalač, 2013). The estimated medium values of the content of crude protein of mushrooms is approximately 20 % of total dry matter (Kalač, 2013).
Therefore, in terms of crude protein, mushrooms are below the contribution made by the animal meats, but is above other products such as milk, some vegetable (*e.g.*, onion and cabbage), fruits (*e.g.*, oranges and apples) or cereals (*e.g.*, rice and corn) (Cheung, 2008). Diferent studies indicate that the content of free amino acids is low (about 1% of dry matter), which means that their nutritional contribution is limited, but still higher than that of most plant proteins and with all the nine essential amino acids required by human body (Cheung, 2008; Ghorai et al., 2009; Kalač, 2009 and 2013).

In addition to their good protein and carbohydrates content, mushrooms present low contents of total fat. Usually these levels vary between 2 and 6 % of dry matter (Kalač, 2009 and 2013). Given the low total fat content, the nutritional contribution of mushroom lipids is limited, what makes them an excellent food choice. In fact, in the present work, the studied species also revealead a low fat content with values that ranged between 0.49 and 4.36 g/100 g dw. Within fatty acids composition mono- and polyunsaturated fatty acids, outweigh saturated fatty acids, as will be describe bellow (Kalač, 2009 and 2013).

The reported values of ash were also fairly low especially in the cultivated samples (1.33 and 11.4 g/100 g dw). In the wild mushrooms the contents were reasonably higher (≈ 5.7 and 29.6 g/100 g dw). Indeed, wild mushrooms are reported as having higher ash contents. This may be due to the greater diversity of the substrate (environment) in mushrooms growing wild, comparing with cultivated species grown in controlled environments and with less variety at

the substrate level (Kalač, 2013). According to literature, the mineral composition (ash) of mushrooms varies between 5 – 12 % of dry matter. The major elements present in mushrooms ash are potassium and phosphorous, being their contents higher or comparable to those found in most vegetables. However, magnesium, calcium and sodium are normally found in lower amounts (Kalač, 2009 and 2013).

Given the low fat content, together with the presence of partially digestible or indigestible carbohydrates, result in low energetic values, an average value of about 370 kcal/100 g dw, making mushrooms low calorie and healthy foods excellent to be inserted in low calorie diets (Kalač, 2009 and 2013). Overall, the studied edible mushrooms revealed low fat contents and quite low energetic values, the present results corroborate the idea that mushrooms seem to be an excellent choice to include in healthy and low calorie diets.

Soluble sugars

Soluble sugars profile was assessed for all the studied mushrooms (except for *Ganoderma lucidum*, which chemical composition have been previously studied). The results are presented in **Table 5**.

Mannitol and trehalose were the main sugars detected in all the studied mushrooms. In fact, these were the only soluble sugars detected in some of the analysed species, such as the mycorrhizal mushrooms, *P. eryngii* (wild species) and *C. militaris*. Actually, regarding *Codyceps* species, some works report the presence of mannitol pointing this polyol as responsible for their bioactive properties, such as diuretic, anti-tussive and anti-free radical activities (Das et al., 2010; Paterson & Russel, 2008). For the other species, the obtained data were variable, cultivated *A. bisporus* (white and brown mushrooms), *F. velutipes*, *L. edodes*, *P. ostreatus* and *P. eryngii* species have, besides mannitol and trehalose, fructose in their chemical constitution. In *P. ostreatus* and *F. velutipes* sucrose was also present. Some authors also have identified other soluble sugars (ribose, xylose, mannose or glucose) in these cultivated species (Kim et al., 2009; Tsai et al., 2009). These differences may be justified by the different substrates used for mushrooms cultivation.

Table 5. Soluble sugars content of the studied edible mushroom species (g/100 g dw; mean ± SD).

Species	Fructose	Mannitol	Sucrose	Trehalose
Cultivated				
Agaricus bisporus (J.E. Lange) Imbach (white mushroom)	0.360 ± 0.001^{i}	64 ± 1^{a}	nd	1.8 ± 0.1^{l}
Agaricus bisporus (J.E. Lange) Imbach (brown mushroom)	0.44 ± 0.02^{h}	49.1 ± 0.9^{b}	nd	2.7 ± 0.2^{k}
Pleurotus ostreatus (Jacq. Ex Fr.) P. Kumm.	0.10 ± 0.02^{k}	5.0 ± 0.2^{h}	0.25 ± 0.02^{b}	40.8 ± 0.2^{b}
Pleurotus eryngii (DC.) Quél.	0.250 ± 0.001^{j}	5.48 ± 0.01^{g}	nd	73 ± 1^{a}
Lentinula edodes (Berk.) Pegler	3.4 ± 0.3^{d}	50 ± 4^{b}	nd	17 ± 1^{e}
Flammulina velutipes (Curtis) Singer	38 ± 2^{a}	8.0 ± 0.6^{e}	0.73 ± 0.02^{a}	22 ± 1^{d}
Cordyceps militaris (L.) Link	nd	2.01 ± 0.08^{l}	nd	24.7 ± 0.8^{c}
Wild				
Amanita caesarea (Scop.) Pers	nd	2.10 ± 0.06^{l}	nd	3.147 ± 0.008^{j}
Cortinarius violaceus (L.: Fr.) Gray	nd	6.35 ± 0.02^{f}	nd	7.9 ± 0.1^{h}
Lactarius volemus (Fr.) Fr.	nd	27 ± 1^{c}	nd	0.931 ± 0.003^{n}
Suillus luteus (L.: Fries) Gray	nd	1.29 ± 0.02^{n}	nd	1.35 ± 0.03^{m}
Agaricus albertii (Bon.)	0.48 ± 0.02^{g}	4.78 ± 0.01^{h}	nd	0.70 ± 0.02^{o}
Agaricus urinascens var. *excellens* (F.H. Møller) Nauta	0.597 ± 0.004^{f}	0.767 ± 0.004^{o}	nd	0.14 ± 0.01^{p}
Pleurotus eryngii (DC.) Quél.	nd	1.40 ± 0.06^{m}	nd	14.2 ± 0.1^{f}
Suillus granulatus (L.) Roussel (Portugal)	4.49 ± 0.01^{c}	3.33 ± 0.05^{i}	nd	4.86 ± 0.03^{i}
Suillus granulatus (L.) Roussel (Serbia)	7.02 ± 0.16^{b}	3.18 ± 0.14^{j}	nd	2.57 ± 0.13^{k}
Leccinum molle Bon (Bon)	3.06 ± 0.01^{de}	11.3 ± 0.1^{d}	nd	2.71 ± 0.04^{k}
Leccinum vulpinum Watling	4.52 ± 0.06^{c}	2.680 ± 0.001^{k}	nd	8.310 ± 0.001^{g}

The different accuracy in the expression of the results is explained by the maintenance of the decimal places allowed by the magnitude of standard deviation. nd- not detected. In each column, different letters mean statistical significant differences among the samples. Significance level was set as $p<0.05$.

Mannitol and trehalose were also present in all the studied wild species. However, in *A. albertii*, *A. urinascens* var. *excellens*, both *S. granulatus* species and both *Leccinum* species, fructose was also detected. Fructose is mostly associated with plants and fruits. In mushrooms, this naturally occurring sugar has important functions, namely as an intermediate for the synthesis of mannitol (Sassoon et al., 2001).

Fatty acids

The results for the fatty acids composition of the studied edible mushrooms are presented in **Table 6**.

As previously referred the main fatty acids found in mushrooms are the saturated palmitic acid (C16:0), the unsaturated oleic acid (C18:1*n*9), and predominantly the polyunsaturated linoleic acid (C18:2*n*6). Even with low lipid contents, mushrooms are quite rich in unsaturated fatty acids, being mushrooms a source of essential fatty acids. The unsaturated fatty acids usually prevailed over saturated fat (Kalač, 2009 and 2013), these were the majority fatty acids found in the studied species, with the exception of *P. linteus*, since the percentage of SFA (58.3%) was the highest fraction comparing with MUFA (14.6%) and PUFA (27.1%), similar results was also reported in on *P. linteus* from China (Deng et al., 2011). Even though, this profile is not so relevant in terms of dietary intake, considering the particularly low fat amounts (0.49 g/100 g dw). Excluding *P. linteus*, in all the other cultivated species PUFA percentages were up to 69.4%, being the great contributor the essential fatty acid linoleic acid. In the wild species both MUFA and/or PUFA are the prevailing fatty acids. One interesting data is regarding cultivated *Pleurotus* species, these mushrooms, obtained under similar conditions, have a fatty very similar acid profile, without significant differences between the percentages of MUFA (13.1 – 13.6%) and PUFA (69.4%).

Table 6. Main fatty acids present in the studied edible mushrooms (relative percentage, mean ± SD).

Species	C16:0	C18:0	C18:1*n*9	C18:2*n*6	SFA	MUFA	PUFA
Cultivated							
Agaricus bisporus (J.E. Lange) Imbach (white mushroom)	11.9 ± 0.1^{f}	3.082 ± 0.001^{h}	1.1 ± 0.3^{o}	77.7 ± 0.3^{c}	20.31 ± 0.03^{f}	1.4 ± 0.3^{q}	78.3 ± 0.3^{c}
Agaricus bisporus (J.E. Lange) Imbach (brown mushroom)	11.07 ± 0.04^{h}	2.96 ± 0.08^{h}	1.3 ± 0.2^{o}	79.41 ± 0.07^{b}	18.4 ± 0.3^{h}	1.8 ± 0.2^{q}	79.82 ± 0.07^{b}
Pleurotus ostreatus Jacq. Ex Fr.) P. Kumm.	11.15 ± 0.09^{gh}	1.60 ± 0.09^{m}	12.7 ± 0.1^{k}	68.9 ± 0.3^{efg}	17.0 ± 0.2^{j}	13.6 ± 0.1^{l}	69.4 ± 0.3^{f}
Pleurotus eryngii (DC.) Quél.	12.8 ± 0.4^{e}	1.7 ± 0.1^{lm}	12.3 ± 0.3^{k}	69 ± 1^{fg}	17.4 ± 0.5^{ij}	13.1 ± 0.5^{l}	69.4 ± 0.9^{f}
Lentinula edodes (Berk.) Pegler	10.28 ± 0.07^{j}	1.58 ± 0.06^{m}	2.3 ± 0.1^{n}	81.1 ± 0.2^{a}	15.09 ± 0.05^{l}	2.9 ± 0.2^{o}	82.0 ± 0.2^{a}
Flammulina velutipes (Curtis) Singer	11.02 ± 0.08^{h}	2.04 ± 0.05^{k}	5.70 ± 0.03^{m}	45.38 ± 0.03^{n}	18.46 ± 0.09^{h}	7.24 ± 0.03^{n}	74.31 ± 0.07^{e}
Cordyceps militaris (L.) Link	12.76 ± 0.03^{e}	5.710 ± 0.001^{c}	7.29 ± 0.03^{l}	68.00 ± 0.06^{fg}	23.40 ± 0.02^{e}	7.73 ± 0.03^{m}	68.87 ± 0.05^{f}
Phellinus linteus (Berkeley & Curtis) Teng	26.8 ± 0.2^{a}	12.3 ± 0.1^{a}	13.5 ± 0.1^{j}	25.3 ± 0.1^{qr}	58.3 ± 0.1^{a}	14.6 ± 0.1^{k}	27.1 ± 0.2^{n}
Wild							
Amanita caesarea (Scop.) Pers	12.4 ± 0.2^{e}	4.9 ± 0.1^{d}	53.8 ± 0.6^{a}	25.7 ± 0.8^{qrs}	19.2 ± 0.2^{g}	54.8 ± 0.6^{a}	26.0 ± 0.8^{n}
Cortinarius violaceus (L.: Fr.) Gray	14.02 ± 0.02^{d}	1.668 ± 0.006^{m}	14.6 ± 0.6^{i}	66.2 ± 0.5^{h}	17.46 ± 0.07^{ij}	16.0 ± 0.6^{j}	66.6 ± 0.5^{g}
Lactarius volemus (Fr.) Fr.	12.2 ± 0.2^{ef}	6.4 ± 0.3^{b}	39.27 ± 0.07^{c}	34.38 ± 0.06^{p}	23.92 ± 0.08^{d}	40.80 ± 0.06^{c}	35.28 ± 0.03^{m}
Suillus luteus (L.: Fries) Gray	10.57 ± 0.02^{i}	2.06 ± 0.03^{k}	31.2 ± 0.1^{e}	52.3 ± 0.3^{l}	14.32 ± 0.09^{n}	33.0 ± 0.2^{e}	52.8 ± 0.3^{k}
Agaricus albertii (Bon.)	11.14 ± 0.03^{gh}	3.12 ± 0.02^{f}	2.0 ± 0.3^{n}	75.7 ± 0.2^{d}	21.14 ± 0.03^{m}	2.4 ± 0.3^{p}	76.5 ± 0.3^{d}
Agaricus urinascens var. *excellens* (F.H. Møller) Nauta	14.9 ± 0.1^{c}	3.572 ± 0.007^{e}	5.5 ± 0.3^{m}	51.2 ± 0.5^{m}	28.79 ± 0.06^{b}	19.2 ± 0.4^{i}	52.0 ± 0.5^{k}
Pleurotus eryngii (DC.) Quél.	17.4 ± 0.1^{b}	4.76 ± 0.03^{d}	47.52 ± 0.03^{b}	24.7 ± 0.1^{qs}	25.79 ± 0.08^{c}	49.05 ± 0.03^{b}	25.2 ± 0.1^{n}
Suillus granulatus (L.) Roussel (Portugal)	9.64 ± 0.02^{k}	3.191 ± 0.002^{g}	24.64 ± 0.06^{g}	57.14 ± 0.09^{j}	15.44 ± 0.05^{k}	26.55 ± 0.02^{g}	58.01 ± 00.07^{i}
Suillus granulatus (L.) Roussel (Serbia)	9.62 ± 0.06^{k}	2.65 ± 0.07^{j}	20.08 ± 0.07^{h}	64.0 ± 0.2^{i}	14.3 ± 0.1^{n}	21.30 ± 0.08^{h}	64.4 ± 0.2^{h}
Leccinum molle Bon (Bon)	11.20 ± 0.08^{gh}	1.791 ± 0.006^{l}	38.6 ± 0.1^{d}	43.49 ± 0.04^{o}	16.9 ± 0.2^{j}	39.3 ± 0.1^{d}	43.79 ± 0.02^{l}
Leccinum vulpinum Watling	12.2 ± 0.1^{ef}	2.78 ± 0.02^{i}	27.06 ± 0.04^{f}	53.5 ± 0.1^{k}	17.1 ± 0.1^{j}	28.61 ± 0.03^{f}	54.3 ± 0.1^{j}

The different accuracy in the expression of the results is explained by the maintenance of the decimal places allowed by the magnitude of standard deviation. Palmitic acid (C16:0); stearic acid (C18:0); oleic acid (C18:1n9); linoleic acid (C18:2n6). SFA- saturated fatty acids; MUFA- monounsaturated fatty acids; PUFA- polyunsaturated fatty acids. In each column, different letters mean statistical significant differences among the samples. Significance level was set as $p<0.05$.

6.2. Mushrooms as a source of bioactive compounds

Given the theme of the present thesis, the edible mushroom species were studied as a source of interesting compounds, as well as matrices with antioxidant, antimicrobial and antitumour potential. Accordingly, in the next sections, the contents in nutrients (*i.e.*, soluble sugars, ascorbic acid, fatty acids and tocopherols) and non-nutrients (*i.e.*, phenolic and other organic acids), as well as the bioactivities are detailed for the studied samples.

6.2.1. Bioactive compounds

Regarding vitamin and pro-vitamin contents, mushrooms are particularly rich in riboflavin (vitamin B_2), niacin (vitamin B_3) and ergocalciferol (vitamin D_2). Thiamine is another vitamin from B complex that is also present in some mushroom species, as well as folic acid and folates (Kalač, 2009 and 2013). Also carotenoids and particularly β-carotene (pro-vitamin A) are also present in mushrooms, even in small quantities (Kalač, 2009 and 2013). Ascorbic acid and tocopherols have been extensively detected in several mushroom species (Heleno et al., 2010; Pereira et al., 2012; Reis et al., 2011b).

Ascorbic acid (Vitamin C)

Although best known for its presence in fruits (mainly citric fruits) and vegetables, vitamin C, namely ascorbic acid, has also been found in fungi (Barros et al., 2007a; Ferreira et al., 2009; Kalač, 2013; Pereira et al., 2012; Reis et al., 2011b). Literature reports that this vitamin is present in small quantities that may vary between 15-30 mg/100g fw (Kalač, 2013).
Ascorbic acid was survey in some of the wild mycorrhizal species under study with values that range 70 and 175 mg/100 g dw, in *C. violaceus* and *A. caesarea*, respectively (**Table 7**).

Table 7. Ascorbic acid content of the studied edible mushroom species (mg/100 g dw; mean ± SD).

Species	Ascorbic acid
Amanita caesarea (Scop.) Pers	175 ± 12^{a}
Cortinarius violaceus (L.: Fr.) Gray	70 ± 8^{c}
Lactarius volemus (Fr.) Fr.	84 ± 3^{b}
Suillus luteus (L.: Fries) Gray	87 ± 10^{b}

The different accuracy in the expression of the results is explained by the maintenance of the decimal places allowed by the magnitude of standard deviation. In each column, different letters mean statistical significant differences among the samples. Significance level was set as $p<0.05$.

As far as we know, there are no reports on ascorbic acid presence in some of these studied species, namely on *C. violaceus* and *S. luteus*. Some authors reported ascorbic acid contents of 0.1 mg/100 g dw in *L. volemus* (Ozen et al., 2011) and 207 mg/100 g dw in *A. caesarea* (Valentão et al., 2005a).

Pereira et al. (2012) studied some Portuguese wild edible mushrooms, reporting lower ascorbic acid levels. Different species have bben studied, namely *Clitocybe gibba* (Pers.) Kumm, *Hygrophorus chrysodon* (Batsch) Fr., *Suillus variegatus* (Sw.) Kuntze, *Boletus impolitus* Fr., *Clavariadelphus pistillaris* (L.) Donk, *Ramaria aurea* (Schaeff.) Quél., *Agaricus campestris* L. ex Fr., *Agaricus lutosus* (F.H. Møller) F.H. Møller, *Leucoagaricus leucothites* (Vittad.) Wasser, *Amanita umbrinolutea* (Secr. ex Gillet), *Clavariadelphus truncatus* (Quél.) Donk, *Cortinarius praestans* (Cordier) Gillet, *Flammulina velutipes* (Curtis) Singer, *Amanita rubescens* (Pers. ex Fr.) Gray, and *Cantharellus cibarius* Fr. The ascorbic acid levels ranging from 0.66 – 33.2 mg/100 g dw (**Table S1**). However, other authors reported higher values for *Calvatia utriformis* (Bull.) Jaap., *Clitopilus prunulus* (Scop. ex Fr.) P. Kumm, *Lycoperdon echinatum* Pers., *Lyophyllum decastes* (Fries: Fries) Singer, *Macrolepiota excoriata* (Schaeff.) M.M. Moser, *Boletus erythropus* (Pers.), *Boletus fragrans* (Vittadini), *Hygrophorus pustulatus* (Persoon : Fries) Fries, *Russula cyanoxantha* (Schaeff.) Fr., and *Russula olivacea* (Schaeff.) Fr., *Armillaria mellea* (Vahl. Ex Fr.) Kummer, *Calocybe gambosa* (Fr.) Donk, and Clitocybe odora (Fr.) P. Kumm. (81 – 400 mg/100 g dw) (Grangeia et al., 2011; Ribeiro et al., 2006; Vaz et al., 2011a; **Table S1**). Another study, reported the

ascorbic acid content in some Portuguese *Boletus* species, from 270 – 532 mg/100 g dw (Heleno et al., 2011; **Table S1**). This variance may be justified by the involvement of ascorbic acid in difference mechanisms related to the ageing process, so the mature stage of the mushroom will have influence on the ascorbic acid levels (Barros et al., 2007b).

Temperature is one of the most reported post-harvest parameters to cause the decrease in the levels of ascorbic acid, as well as the heating time. Though temperature influences the amounts of this vitamin in food, low temperatures do not appear to greatly affect the natural decrease of ascorbic acid resulting from processing. However, the losses during freeze-drying (used in our samples) seem to be much lower comparing to air-drying treatments (Veras et al., 2012). Actually, many studies have been carried out on several matrices, concluding that the low temperatures and residual moisture contents of the lyophilisation process can inhibit a degradation of ascorbic acid (Veras et al., 2012). Some studies show that the ascorbic acid content in lyophilized wild mushrooms may be quite low (0.66 – 33.16 mg/100 g dw; Pereira et al., 2012), while other species have a range between 81.32 – 400.36 mg/100 g dw (Grangeia et al., 2011).

Other Organic acids

In addition to their functions in primary metabolism, some of the organic acids mainly found in mushrooms have also biological activity. Citric and quinic acids are known for their antioxidant potential (Choe & Min, 2009; Hung et al., 2006). Fumaric acid has been referred as effective against psoriasis and inflammation and as potentially neuro- and chemoprotector (Altmeyer et al., 1994; Baati et al., 2011). Malic acid has bactericidal effects (Raybaudi-Massilia et al., 2009), being employed in food additives as well as excipient in pharmaceutical and polymer industries (Nagarajkumar et al., 2005). While oxalic acid is also uses as excipient in pharmaceutical (Lian et al., 1999), it's widely distributed in plant foods, mainly greens and vegetables. A high oxalic acid consumption in the diet could be associated with toxic effects, this compound is associated with calcium oxalate monohydrate crystals formation that are accumulated in the urinary system, which are associated with renal failure (Guo & McMartin, 2005) and kidney stones (Taylor & Curhan, 2007). In addition, oxalic acid is assumed to have a negative impact on mineral absorption due to the ability to bind free minerals in the small intestine, forming insoluble oxalates that remain non-absorbed in the gut. Generally, oxalic

acid may reduce calcium absorption in about 1/6, so foods with a ratio of oxalic acid/Ca lower than 2.5 are preferably for human diet (Mahan et al., 2012). Although oxalate is present in numerous mushroom species, their levels are much lower than the others found in several vegetables, such as chard, spinach, among others. Moreover, oxalate is poorly absorbed. It has been estimated that 2 – 12% of the oxalate ingested is absorbed, the higher percentage when consumed during fasting (Noonan & Savage, 1999) and the minimal poison dose for humans is proximately 5 g per day for an adult (Guil et al., 1997).

The amounts of organic acids present in the studied edible mushrooms are presented in **Table 8**. There was not a main organic acid for all studied mushrooms. In general terms, malic acid was found in higher amounts in *A. albertii* (1.39 g/100g dw) and *P. eryngii* (6.1 g/100g dw), and citric acid in *C. militaris* (8 g/100g dw), *L. molle* (2.62 g/100g dw) and *S. granulatus* (from Serbia; 1.77 g/100g dw). *C. militaris*, the studied cultivated species, revealed the presence of oxalic acid (0.334 g/100 g dw), citric acid (8.0 g/100 g dw) and fumaric acid (0.13 g/100 g dw). In some of the wild species, besides these three acids, it was possible to quantify also quinic and malic acids. It should be noted that the chemistry of an organism may also vary according to the conditions under which it develops (Hanson, 2008). This difference between cultivated and wild samples may be due to the environmental conditions. Moreover, once the organic acids are constituents of the Krebs cycle, and being wild mushrooms more prone to adverse conditions, these may have higher energy requirements comparing to mushrooms growing under controlled conditions. Interestingly, in *S. granulatus*, some acids were only obtained in the Portuguese species (quinic acid), and others in the Serbian one (malic and citric acids). These results support the idea that the chemical content among species from different sources can be similar, as a characteristic of the species, but the chemical profile may vary between them. Other authors dentified in *S. granulatus* oxalic, aconitic, citric, malic, quinic, succinic, shikimic and fumaric acids, with succinic and shikimic acids appearing in lower quantities (Ribeiro et al., 2006 and 2008b; **Table S1**). Although morphologically similar, fungi metabolites may be very different. Some metabolites may be produced by all the varieties of a particular species, while others may be specific metabolites of an organism.

Although similar quantities of organic acids have been found in several species of edible mushrooms (**Table S1**), some of them produce considerably higher quantities of total organic

acids than those found in the present work. This is the case of *Agaricus comtulus* Fr. (13 g/100 g dw), *Boletus aereus* Bull. (11 g/100 g dw) *Boletus armeniacus* Quél. (18 g/100 g dw), *Clitocybe odora* (Fr.) P.Kumm. (22 g/100 g dw), *Lentinula edodes* (Berk.) Pegler (21 g/100 g dw), *Lepista nuda* (Bull.) Cooke (18 g/100 g dw), *Marasmius oreades* (Bolton) Fr (14 g/100 g dw), and *Sarcodon imbricatus* (L.) P.Karst. (25 g/100 g dw) (Barros et al., 2013; **Table S1**).

Table 8. Organic acids content of the studied edible mushroom species (g/100 g dw; mean ± SD)

Species	Oxalic acid	Quinic acid	Malic acid	Citric acid	Fumaric acid
Cultivated					
Cordyceps militaris (L.) Link	0.334 ± 0.001^{e}	nd	nd	8.0 ± 0.1^{a}	0.13 ± 0.03^{fg}
Wild					
Agaricus albertii (Bon.)	0.623 ± 0.003^{c}	0.65 ± 0.02^{a}	1.39 ± 0.02^{b}	0.71 ± 0.02^{d}	0.1551 ± 0.0003^{fg}
Agaricus urinascens var. *excellens* (F.H. Møller) Nauta	0.865 ± 0.001^{b}	nd	0.76 ± 0.02^{d}	0.4956 ± 0.0004^{e}	0.0978 ± 0.0004^{g}
Pleurotus eryngii DC.) Quél.	0.246 ± 0.005^{g}	nd	6.1 ± 0.1^{a}	0.426 ± 0.001^{f}	0.5469 ± 0.0001^{d}
Suillus granulatus (L.) Roussel (Portugal)	3.35 ± 0.08^{a}	0.36 ± 0.01^{b}	nd	nd	0.923 ± 0.001^{c}
Suillus granulatus (L.) Roussel (Serbia)	0.42 ± 0.02^{d}	nd	0.94 ± 0.16^{c}	1.77 ± 0.25^{c}	1.31 ± 0.03^{a}
Leccinum molle Bon (Bon)	0.29 ± 0.01^{f}	nd	nd	2.62 ± 0.07^{b}	1.0387 ± 0.0007^{b}
Leccinum vulpinum Watling	0.115 ± 0.001^{h}	0.133 ± 0.002^{c}	nd	nd	0.2501 ± 0.0001^{e}

The different accuracy in the expression of the results is explained by the maintenance of the decimal places allowed by the magnitude of standard deviation. nd- not detected. In each column, different letters mean statistical significant differences among the samples. Significance level was set as $p<0.05$.

Phenolic acids and related compounds

Phenolic compounds, namely phenolic acids, are reported in literature as one of the main antioxidants found in mushrooms all over the world (Ferreira et al., 2009; Kim et al., 2008; Nowacka et al., 2015; Yahia et al., 2017).

Phenolic compounds are secondary metabolites produced in response to the environmental conditions, such as light or pollution, protecting mushrooms from the different biotic and abiotic stresses. So, the obtained results both for *in vitro* and *in vivo* species may be related with different responses of the species to the surroundings. Hereupon, *in vitro* culture may be a method for obtaining bioactive compounds. This methodology can be optimized, for instance by increasing the stress conditions (*e.g.*, placing the cultures in light).

In general the profile of phenolic compounds varied greatly depending on the species; however, *p*-hydroxybenzoic acid and cinnamic acids were detected in almost all samples, highlighting *A. albertii*, *A. urinascens* and *P. eryngii* by presenting the highest contents of these two bioactive compounds (**Table 9**). Regarding the most widely appreciated cultivated mushrooms, the phenolic contents were variable, being *A. bisporus* the species with the highest contents of total phenolic acids (≈ 6.6 mg/100 g dw). As previous referred, the mycelium of these species (*A. bisporus*, *L. edodes*, *P. ostreatus* and *P. eryngii*) was produced through *in vitro* techniques (data not shown in this section), and the profiles differed slightly between species and even within species.

About the wild samples, it was possible to quantify one or more phenolic acids in the studied mushrooms. Generally, wild mushroom presented higher content of phenolic acids comparing to cultivated species. This is because these species, into the wild, may be subject to greater stress conditions. Thus, it is normal that some compounds are found in certain species and not in others, just as the same species may have different profiles depending on the nature of its growth.

Some of the studied Portuguese wild edible species studied in the present work, have been also studies for phenolic compounds composition by other authors. Barros et al. (2009) identified only *p*-hydroxybenzoic acid (2.6 mg/100 g dw) in *A. bisporus*, phenolic acid not identified by us in both *A. bisporus* species studied. Fernandes et al. (2015), reported the presence of only *p*-hydroxybenzoic acid (3.9 mg/100 g dw) and the related compound

cinnamic acid (2.48 mg/100 g dw) in *A. caesarea* (**Table S1**). There are also some reports with the studied *Suillus* species. *S. granulatus* and *S. luteus* have been studied by Ribeiro et al. (2006), and both phenolic profiles have been reported as constituted only by quercetin (0.2 – 1.59 mg/100 g dw for *S. granulatus* and 0.46 mg/100 g dw for *S. luteus*; **Table S1**).

Table 9. Phenolic acids (and related compounds) content of the studied edible mushroom species (mg/100 g dw; mean ± SD).

Species	Gallic acid	Protocatechuic acid	*p*-Hydroxybenzoic acid	*p*-Coumaric acid	Cinnamic acid
Cultivated					
Agaricus bisporus (J.E. Lange) Imbach (white mushroom)	6.3 ± 0.6^{a}	nd	nd	0.231 ± 0.003^{c}	0.038 ± 0.001^{k}
Agaricus bisporus (J.E. Lange) Imbach (brown mushroom)	nd	nd	nd	nd	0.0094 ± 0.0001^{o}
Pleurotus ostreatus (Jacq. Ex Fr.) P. Kumm.	nd	0.077 ± 0.001^{d}	0.156 ± 0.003^{h}	0.081 ± 0.002^{e}	0.023 ± 0.001^{m}
Pleurotus eryngii (DC.) Quél.	nd	0.006 ± 0.001^{f}	0.010 ± 0.002^{m}	0.104 ± 0.002^{d}	0.0195 ± 0.0006^{n}
Lentinula edodes (Berk.) Pegler	nd	0.0364 ± 0.0004^{e}	0.157 ± 0.007^{h}	nd	0.00194 ± 0.00006^{p}
Cordyceps militaris (L.) Link	nd	nd	0.021 ± 0.001^{l}	nd	0.1060 ± 0.0006^{j}
Wild					
Amanita caesarea Scop.) Pers	nd	0.67 ± 0.03^{a}	0.72 ± 0.01^{e}	nd	0.0310 ± 0.0005^{l}
Cortinarius violaceus (L.: Fr.) Gray	nd	nd	1.30 ± 0.05^{d}	nd	0.565 ± 0.009^{d}
Lactarius volemus (Fr.) Fr.	nd	nd	0.46 ± 0.04^{g}	nd	0.321 ± 0.001^{f}
Suillus luteus (L.: Fries) Gray	nd	0.468 ± 0.006^{b}	nd	nd	0.41 ± 0.01^{e}
Agaricus albertii (Bon.)	nd	nd	8.2 ± 0.3^{a}	3.5 ± 0.3^{a}	2.51 ± 0.04^{a}
Agaricus urinascens var. *excellens* (F.H. Møller) Nauta	nd	nd	3.3 ± 0.2^{c}	1.3 ± 0.1^{b}	1.8 ± 0.1^{b}
Pleurotus eryngii (DC.) Quél.	nd	nd	3.8 ± 0.2^{b}	nd	0.854 ± 0.004^{c}
Suillus granulatus (L.) Roussel (Portugal)	0.105 ± 0.006^{b}	nd	0.4828 ± 0.0005^{f}	nd	0.1271 ± 0.0003^{i}
Suillus granulatus (L.) Roussel (Serbia)	nd	nd	0.13 ± 0.01^{i}	nd	0.03 ± 0.001^{n}
Leccinum molle Bon (Bon)	nd	nd	0.060 ± 0.002^{k}	nd	0.1313 ± 0.0004^{h}
Leccinum vulpinum Watling	0.0831 ± 0.0006^{c}	0.35 ± 0.02^{c}	0.090 ± 0.001^{j}	nd	0.1720 ± 0.0004^{g}

The different accuracy in the expression of the results is explained by the maintenance of the decimal places allowed by the magnitude of standard deviation. nd- not detected. In each column, different letters mean statistical significant differences among the samples. Significance level was set as $p<0.05$.

Vitamin E – Tocopherols

Regarding tocopherols, as shown in **Table 10**, we could not detect an isoform prevalent in all the species.

Generally, cultivated species revealed lower values of total tocopherols (≈ 2 – 56 μg/100 g dw) comparing with wild species (≈ 25 – 528 μg/100 g dw). Since tocopherols have antioxidant properties, their production could be higher when mushrooms are under stress conditions. Therefore, the controlled production of mushrooms (cultivated species) may not allowed the production of high quantites of tocopherols. Not all the analyzed isoforms were characterized in the selected mushroos, the wild *Agaricus* species did not revealed any isoform of vitamin E and the cultivated *C. militaris* only presented α-tocopherol. This may have been due effectively to its absence, or due to being degraded, once vitamin E is very sensitive to heat and light. Furthermore, there is not a major isoform for all analyzed mushrooms, β-tocopherol was the main isoform found in *S. granulatus* (from both origins), γ-tocopherol in *L. vulpinum* and *S. luteus* (297 and 337 μg/100 g dw, respectively), while δ-tocopherol was the main isoform found in *A. caesarea* (74.3 μg/100 g dw).

In mushrooms, according with literature, the content in tocopherols is usually between 0.05 – 0.3 mg/100 g dw, levels quite lower than those found in vegetables (Kalač, 2013).

It is possible to find in literature other similar works including different species of Portuguese wild edible mushrooms. In those studies, which include species belonging to different genus, such as *Amanita*, *Boletus*, *Laccaria*, *Lactarius*, *Lepista*, *Russula* or *Suillus* species, β-tocopherol (Heleno et al., 2010; Reis et al., 2014a and 2014b) and γ-tocopherol (Leal et al., 2013; Reis et al., 2011b and 2012a, Vaz et al., 2011a), seems to be the prevailing isoforms, which is according with our results.

Table 10. Tocopherols composition of the studied edible mushroom species (μg/100 g dw; mean ± SD).

Species	α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol	Total tocopherols
Cultivated					
Agaricus bisporus (J.E. Lange) Imbach (white mushroom)	0.23 ± 0.01^{kj}	0.85 ± 0.01^{i}	1.51 ± 0.09^{i}	2.6 ± 0.1^{g}	5.18 ± 0.02^{m}
Agaricus bisporus (J.E. Lange) Imbach (brown mushroom)	0.28 ± 0.05^{j}	0.71 ± 0.07^{j}	1.1 ± 0.2^{j}	2.54 ± 0.07^{g}	4.6 ± 0.3^{n}
Pleurotus ostreatus (Jacq. Ex Fr.) P. Kumm.	0.59 ± 0.05^{i}	nd	1.4 ± 0.2^{ijh}	1.6 ± 0.1^{h}	3.6 ± 0.3^{o}
Pleurotus eryngii (DC.) Quél.	0.251 ± 0.007^{j}	2.16 ± 0.01^{h}	1.8 ± 0.1^{h}	0.62 ± 0.02^{i}	4.9 ± 0.2^{n}
Lentinula edodes (Berk.) Pegler	0.92 ± 0.03^{h}	nd	5.5 ± 0.4^{g}	4.4 ± 0.4^{f}	10.83 ± 0.07^{l}
Flammulina velutipes (Curtis) Singer	0.19 ± 0.02^{l}	nd	1.62 ± 0.02^{hi}	nd	1.81 ± 0.01^{p}
Cordyceps militaris (L.) Link	56 ± 2^{a}	nd	nd	nd	56 ± 2^{i}
Phellinus linteus Berkeley & Curtis) Teng	27.2 ± 0.5^{b}	nd	20 ± 2^{e}	nd	48 ± 3^{j}
Wild					
Amanita caesarea (Scop.) Pers	7.0 ± 0.2^{g}	nd	46 ± 1^{c}	74.3 ± 0.3^{b}	127.3 ± 0.9^{f}
Cortinarius violaceus L.: Fr.) Gray	8.3 ± 0.6^{fg}	26 ± 2^{e}	258 ± 14^{b}	55 ± 2^{d}	348 ± 19^{c}
Lactarius volemus (Fr.) Fr.	$7.5 \pm 0.6f^{g}$	nd	31 ± 1^{d}	69 ± 2^{c}	108 ± 3^{g}
Suillus luteus (L.: Fries) Gray	19 ± 2^{c}	15 ± 1^{f}	337 ± 29^{a}	79 ± 3^{b}	450 ± 35^{b}
Agaricus albertii (Bon.)	nd	nd	nd	nd	nd
Agaricus urinascens var. *excellens* (F.H. Møller) Nauta	nd	nd	nd	nd	nd
Pleurotus eryngii (DC.) Quél.	7 ± 1^{fg}	48 ± 6^{d}	32 ± 5^{d}	nd	87 ± 2^{h}
Suillus granulatus (L.) Roussel (Portugal)	17.9 ± 0.5^{c}	175 ± 2^{c}	nd	102 ± 3^{a}	295 ± 5^{d}
Suillus granulatus (L.) Roussel (Serbia)	6.8 ± 0.3^{g}	179.7 ± 0.6^{b}	13.6 ± 0.9^{f}	19.8 ± 0.3^{e}	219.9 ± 0.8^{e}
Leccinum molle Bon (Bon)	12.5 ± 0.3^{e}	12.9 ± 0.5^{g}	nd	nd	25.4 ± 0.1^{k}
Leccinum vulpinum Watling	14.8 ± 0.5^{d}	216 ± 10^{a}	297 ± 26^{ab}	nd	528 ± 34^{a}

The different accuracy in the expression of the results is explained by the maintenance of the decimal places allowed by the magnitude of standard deviation. nd- not detected. In each column, different letters mean statistical significant differences among the samples. Significance level was set as $p<0.05$.

6.3. Evaluation of the antioxidant potential of mushrooms

The results for the antioxidant potential of the studied mushrooms are presented in **Table 11**. All the studied samples revealed antioxidant potential. Regarding cultivated samples, *C. militaris* revealed antioxidant potential mainly through lipid peroxidation inhibition (lowest EC_{50} values for β-carotene/linoleate and TBARS assays, between 0.77 – 1.05 mg/mL). The remaining cultivated species seems to have antioxidant potential maily through their reducing power capacity and lipid peroxidation inhibition, since the higher EC_{50} values were obtained for the DPPH radical-scavengung activity assay (2.28 – 12.2 mg/mL). Although, *P. linteus* have been also studied for this activity, different fractions (polysaccharides, glucans and triterpenoids) were analysed from this mushroom, and then compared with the methanolic and ethanolic extract. Therefore, for this integrative discussion, this species will not be taking into account (see article number 7, in Results section). However, it has been concluded that *P. linteus* proved to have high potential for antioxidant purposes. Among the assayed fractions, glucans showed the lowest antioxidant activity. The highest activity among assays was obtained for TBARS formation inhibition, while the worst values resulted from β-carotene bleaching inhibition. Neverthless, the methanolic extract of *P. linteus* revealed the lowest EC_{50} values for DPPH radical-scavenging activity (70 µg/mL), reducing power (50.5 µg/mL) and lipid peroxidation inhibition, either for β-carotene bleaching inhibition (114 µg/mL) as well as for TBARS inhibition (8 µg/mL).

Concerning the wild species, it seems that they have better antioxidant properties (generally, lower EC_{50} values). However, these results are not so linear and unbending. In general terms, S. granulatus (from both origins) and *L. vulpinum* presented the better results in all antioxidant assays, comparing with the other wild species, while *S. luteus* also revealed good results for the reducing power and β-carotene bleaching inhibition assays. When we compare samples from the same genus (*A. albertii* and *A. urinascens* var. *excellens*) or from the same species from different origins (*S. granulatus*), we can see statistically significant differences in almost all the assays. This result would be expected, since the chemical profiles previous analysed also varied.

Since species with higher content of antioxidant compounds, such as tocopherols (*L. vulpinum, S. luteus* or *S. granulatus* from Portugal) or phenolic compounds (*A. albertii*), did not reveal a differentiating antioxidant activity, a synergistic action between the explored compounds and others may be inherent to this property.

Barros et al. (2008a) described the antioxidant potential of *A. bisporus*, reporting EC_{50} values higher than those found by us. The authors obtained EC_{50} values of 3.63 mg/mL for reducing power, 9.61 mg/mL for the DPPH radical-scavenging activity, 21.39 mg/mL for β-carotene bleaching inhibition and 46.82 mg/mL for TBARS inhibition. Fernandes et al. (2015) reported for *A. caesarea* similar EC_{50} values specially concerning the reducing power (1.36 mg/mL) and for the DPPH radical-scavenging activity (8.0 mg/mL). The aqueous extract of *S. granulatus* has been studied by Ribeiro et al. (2008) for its antioxidant potential. However, as the authors reported the DPPH radical-scavenging activity as IC_{25} values (0.184 – 196 mg/mL), we cannot compare directly with ours (**Table S1**). A methanol / water extract obtained from *S. luteus* has been studied for its antioxidant potential, revealing EC_{50} values similar to ours (0.97 mg/mL for reducing power, 2.86 mg/mL for DPPH radical-scavenging activity and 1.64 mg/mL for β-carotene bleaching inhibition) (Ribeiro et al., 2015; **Table S1**).

Therefore, although the present study discloses that mushrooms are indeed natural matrices with antioxidant properties, further studied need to be performed in order to identify the compounds effectively responsible for this activity. For instance, it is possible to fractionate the studied extracts, and to charcaterize the most bioactive fractions, in order to verify the compounds present in these.

Table 11. Antioxidant potential of the studied edible mushroom species (mean ± SD).

Species	Assay				
	Reducing Power		Radical scavenging activity	Lipid peroxidation inhibition	
	Folin-Ciocalteu assay	Ferricyanide/Prussian blue assay	DPPH radical-scavenging activity assay	β-carotene/linoleate assay	TBARS assay
Cultivated					
Agaricus bisporus (J.E. Lange) Imbach (white mushroom)	23.3 ± 0.3^{e}	0.81 ± 0.02^{g}	3.13 ± 0.09^{j}	3 ± 1^{gh}	2.9 ± 0.5^{c}
Agaricus bisporus (J.E. Lange) Imbach (brown mushroom)	37.3 ± 0.2^{c}	1.46 ± 0.04^{h}	2.28 ± 0.06^{k}	4.8 ± 0.2^{de}	1.45 ± 0.08^{d}
Pleurotus ostreatus (Jacq. Ex Fr.) P. Kumm.	12.5 ± 0.2^{f}	3.31 ± 0.02^{c}	6.5 ± 0.2^{i}	2.7 ± 0.2^{hi}	2.6 ± 0.9^{c}
Pleurotus eryngii (DC.) Quél.	7 ± 2^{g}	3.72 ± 0.07^{b}	8.7 ± 0.1^{g}	4.7 ± 0.6^{ef}	3.9 ± 0.6^{b}
Lentinula edodes (Berk.) Pegler	8.8 ± 0.9^{g}	2.62 ± 0.04^{e}	6.4 ± 0.7^{hi}	3.9 ± 0.3^{fg}	1.6 ± 0.4^{d}
Cordyceps militaris (L.) Link	15.8 ± 0.5^{f}	5.55 ± 0.03^{a}	12.2 ± 0.7^{d}	1.05 ± 0.07^{j}	0.77 ± 0.03^{ef}
Wild					
Amanita caesarea (Scop.) Pers	-	1.8 ± 0.1^{g}	7.4 ± 0.2^{hi}	8.1 ± 0.3^{c}	-
Cortinarius violaceus (L.: Fr.) Gray	-	2.35 ± 0.07^{e}	15.7 ± 0.4^{b}	5.7 ± 0.2^{d}	-
Lactarius volemus (Fr.) Fr.	-	2.4 ± 0.2^{e}	21.7 ± 0.2^{a}	9.1 ± 0.03^{b}	-
Suillus luteus (L.: Fries) Gray	-	0.74 ± 0.02^{i}	1.93 ± 0.04^{l}	0.61 ± 0.01^{k}	-
Agaricus albertii (Bon.)	18.36 ± 0.09^{f}	3.04 ± 0.02^{d}	10.2 ± 0.2^{e}	3.8 ± 0.4^{g}	0.6 ± 0.3^{fg}
Agaricus urinascens var. *excellens* (F.H. Møller) Nauta	15.4 ± 0.3^{f}	3.71 ± 0.03^{b}	13.8 ± 0.2^{c}	4.3 ± 0.3^{efg}	6.6 ± 0.9^{a}
Pleurotus eryngii (DC.) Quél.	18 ± 5^{ef}	2.18 ± 0.04^{ef}	9.21 ± 0.05^{f}	15 ± 1^{a}	1.59 ± 0.02^{d}
Suillus granulatus (L.) Roussel (Portugal)	40.8 ± 0.8^{b}	0.569 ± 0.009^{j}	0.98 ± 0.02^{n}	0.45 ± 0.08^{m}	0.033 ± 0.001^{g}
Suillus granulatus (L.) Roussel (Serbia)	44.4 ± 0.3^{a}	0.412 ± 0.006^{l}	0.89 ± 0.02^{o}	0.48 ± 0.05^{l}	0.167 ± 0.007^{g}
Leccinum molle Bon (Bon)	23.1 ± 0.2^{ef}	2.127 ± 0.002^{f}	10.7 ± 0.5^{e}	2.4 ± 0.2^{i}	1.3 ± 0.2^{de}
Leccinum vulpinum Watling	35.4 ± 0.7^{d}	0.541 ± 0.0065^{k}	1.19 ± 0.01^{m}	0.15 ± 0.07^{n}	0.279 ± 0.004^{g}

The different accuracy in the expression of the results is explained by the maintenance of the decimal places allowed by the magnitude of standard deviation. (-) not performed. Concerning the *Folin-Ciocalteu* assay, higher values mean higher reducing power; for the other assays, the results are presented in EC_{50} values, which means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: extract concentration corresponding to 50% antioxidant activity or 0.5 absorbance for the ferricyanide/Prussian blue assay. In each column, different letters mean statistical significant differences among the samples. Significance level was set as $p<0.05$.

6.4. Evaluation of the antimicrobial potential of mushrooms

The antimicrobial potential was assessed for the cultivated *C. militaris*, as well as for the wild *S. granulatus* species (from Portugal and Serbia). As for the antioxidant potential, *P. linteus* have been also studied for this activity (different fractions, compared with different extracts; see article number 7 from Results section). Once again, the methanolic extract was the one that showed the best results, comparing with the ethanolic extract and with the different fractions. Regarding these latter, the glucans fraction was the less effective against bacterial growth, except in the case of *Salmonella typhimurium*. Furthermore, the fractions with highest antifungal activity were glucans and triterpenoids (which were actually the least effective against bacteria), presenting lower MIC and MFC values than bifonazole and ketoconazole on *Aspergillus versicolor*, *Aspergillus ochraceus*, *Trichoderma viride* and *Penicillium funiculosum*. *A. versicolor*, *A. ochraceus* and *T. viride* (MIC < 0.27 mg/mL; MFC < 0.77 mg/mL, for all samples) were the fungal species with highest susceptibility to *P. linteus*.

However, as previously referred, for this integrative discussion, only *C. militaris* and *S. granulatus* will be taking into account.

Figure 37 to Figure 40 represent the minimum inhibition concentration (MIC) for bacteria and fungi, minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) for the studied mushroom species. Regarding bacteria, the mushroom methanolic extracts were tested against *Staphylococcus aureus*, *Bacillus cereus*, *Micrococcus flavus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli* and *Enterobacter cloacae*. While, the selected fungi for the antifungal assays were *Aspergillus fumigatus*, *Aspergillus versicolor*, *Aspergillus ochraceus*, *Aspergillus niger*, *Trichoderma víride*, *Penicillium funiculosum*, *Penicillium ochrochloron* and *Penicillium verrucosum* var. *cyclopium*.

According the literature, mushrooms generally exhibit higher antibacterial effects against Gram-positive bacteria (Alves et al., 2012). In the present work, all the species revealed antimicrobial properties against both Gram-positive (*Staphylococcus aureus*, *Bacillus cereus*, *Micrococcus flavus* and *Listeria monocytogenes*) and Gram-negative (*Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli* and *Enterobacter cloacae*) bacteria, with no apparent trend.

Regarding antibacterial MIC's (**Figure 37**), samples revealed very interesting results even with lower MIC values (0.05 – 0.2 mg/mL) than the antibiotics streptomycin and ampicillin (0.04 – 0.74). Except in the case of *C. militaris*, which revealed values much higher than the previous ones (0.015 – 3.0 mg/mL).

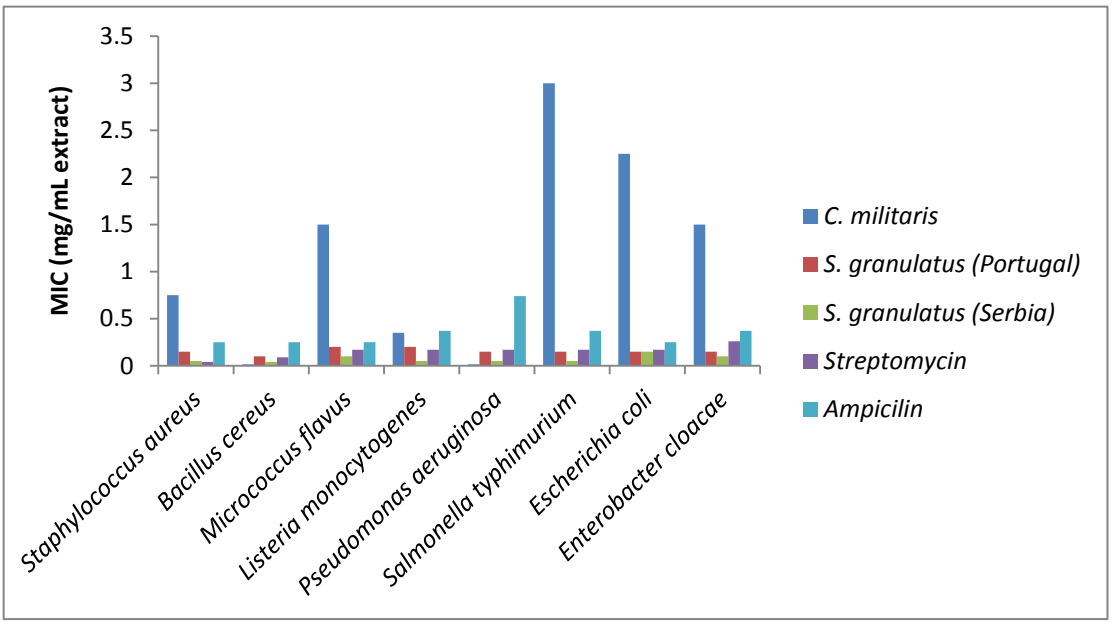

Figure 37. Antibacterial MIC's (mg/mL of extract).

Regarding the antibacterial MBC's (**Figure 38**), the trend remained; both *S. granulatus* species presented lower values (0.05 – 0.4 mg/mL) than *C. militaris* (0.03 – 3.0 mg/mL) and even than commercial antibiotics (0.09 – 0.74 mg/mL).

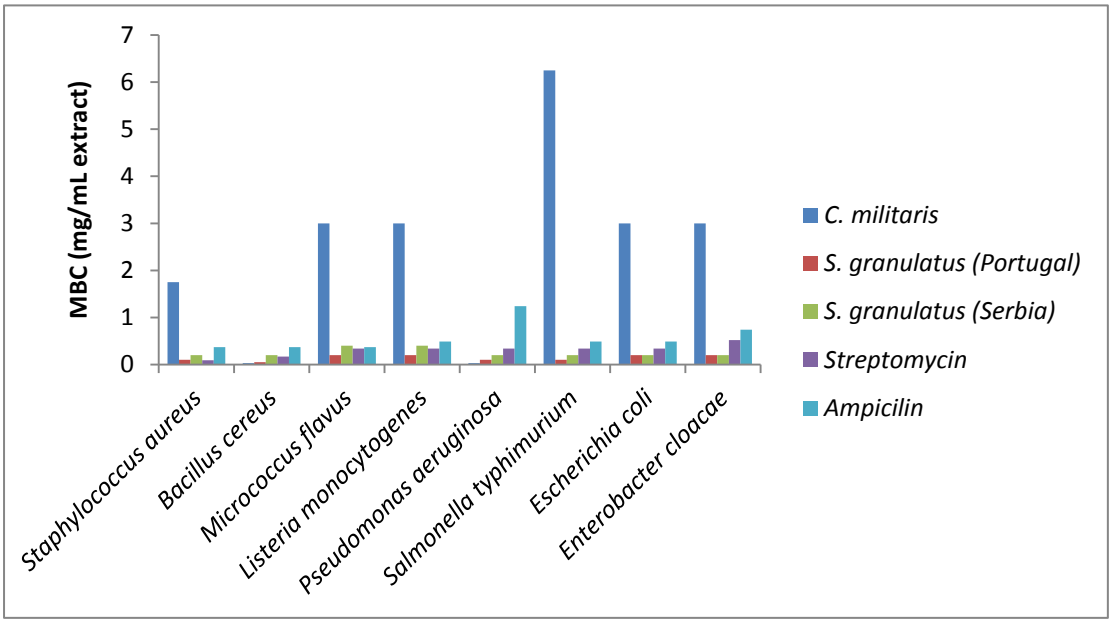

Figure 38. Antibacterial MBC's (mg/mL of extract).

As previously referred, the extracts were also tested against different fungi strains. Regarding antifungal MIC's, *C. militaris* revealed the better results against *Aspergillus fumigatus* (0.04 mg/mL), *Aspergillus versicolor* (0.04 mg/mL) and *Aspergillus ochraceus* (0.04 mg/mL) even when compared with the antifungal drugs bifonazole (0.15 mg/mL) and ketoconazole (0.2 mg/mL). However, it presented the highest MIC's against *Aspergillus niger* (0.75 mg/mL) and *Penicillium verrucosum* var. *cyclopium* (6.25 mg/mL) (**Figure 39**).

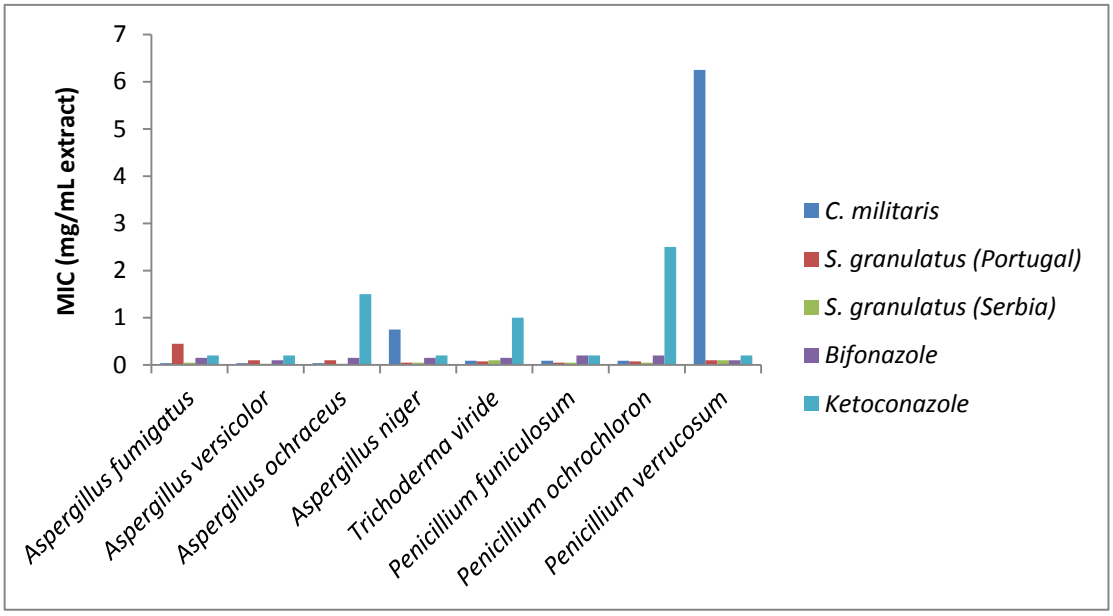

Figure 39. Antifungal MIC's (mg/mL of extract).

Regarding the antifungal MFC's, the better results were presented by *S. granulatus*, mainly the Serbian species (0.05 – 0.2 mg/mL). As in the antibacterial activity, *C. militaris* presented the highest MFC values, specially against *Aspergillus fumigatus* (6.25 mg/mL), *Aspergillus versicolor* (1.5 mg/mL), *Aspergillus ochraceus* (6.25 mg/mL), *Aspergillus niger* (6.25 mg/mL), and *Penicillium verrucosum* var. *cyclopium* (12.5 mg/mL) (**Figure 40**).

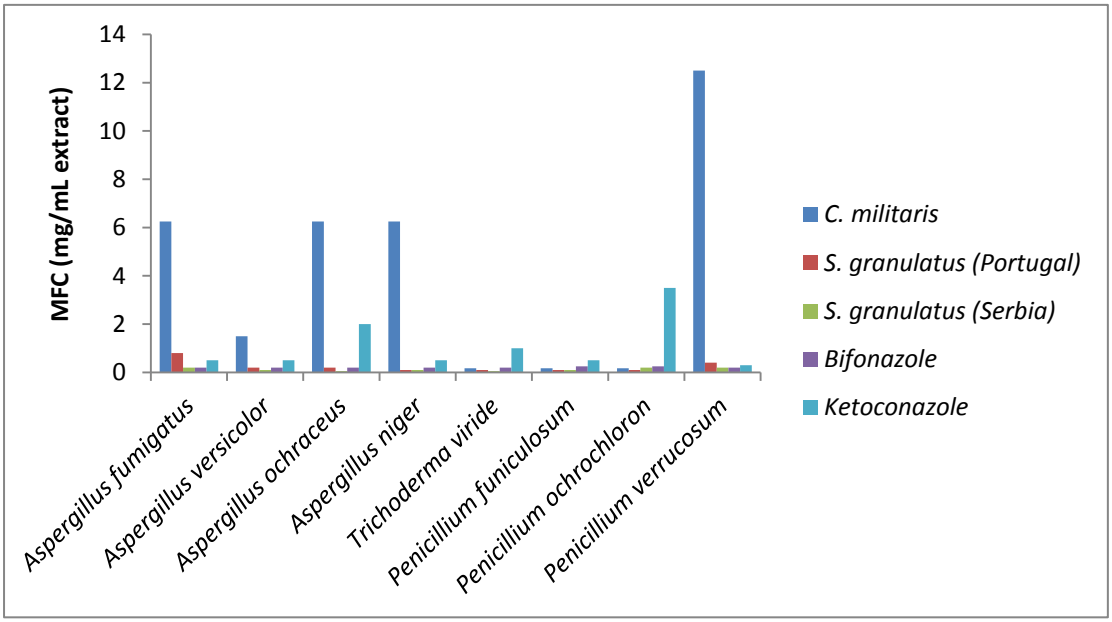

Figure 40. Antifungal MFC's (mg/mL of extract).

Although *C. militaris* revealed the highest MIC's, MBC's and MFC's against almost all bacteria and fungi strains, its antimicrobial potential (or the antimicrobial potential of its constituints) has been proved along the years. For example, Park et al. (2009) studied the antifungal potential of a protein extracted from this mushroom species against *Fusarium oxysporum* and *Botrytis cinerea*, using the disc plate diffusion assay. The authors obtained good results especially for *Fusarium oxysporum*. Wong et al. (2011), also studied the antifungal activity of cordymin (an antifungal peptide obtained from *C. militaris*), reporting IC_{50} values of 50 μM, 10 μM, 80 μM, and 0.75 mM for the inhibition of mycelial growth of *Bipolaris maydis*, *Mycosphaerella arachidicola*, *Rhizoctonia solani* and *Candida albicans*, respectively. Tuli et al. (2014) studied the antimicrobial activity of *C. militaris* fractions (hexane; chloroform; *n*-butanol; aqueous) and cordycepin, a nucleoside derivative, testing them against pathogenic bacteria (*Staphylococcus aureus* and *Klebsiella pneumoniae*) and fungi (*Aspergillus niger* and *Trichophyton rubrum*). Using also the disc plate diffusion assay, the authors concluded that both extracts of *C. militaris* and cordycepin, revealed a broad-spectrum of activity against both gram-positive and gram-negative bacteria and fungi as well. The authors stated also that the antimicrobial activity is directly correlated with the content of cordycepin.

Overall, the studied species revealed antibacterial and antifungal antivities. Since *S. granulatus* and *C. militaris* shown higher values of some of the organic acids (*i.e.*, oxalic acid, citric acid and fumaric acid), these may be contributing for the antimicrobial potential of these mushrooms, as reported in literature (Mani-Lopez et al., 2012; Theron & Lues, 2011). However, as for other bioactivities, and as for many natural matrices, other compounds, such as phenolic compounds, may contribute for this behaviour in a synergistic way.

The antimicrobial potential of the studied mushrooms against different bacteria and fungi strains could be useful to discover new alternative to the existing antimicrobial agents, as well as constitute alternatives to synthetic chemicals with application in agriculture, food or pharmaceutical industry.

6.5. Evaluation of the antitumor potential of mushrooms

6.5.1. Antiproliferative effects

Some of the mushroom species were also studied for their antitumour potential (*C. militaris*, *L. vulpinum* and *G. lucidum*). For those samples, the extracts were tested against a panel of human tumour cell lines, in order to verify their growth inhibitory capacity. All the tested species exhibited cytotoxicity effects in the studied human tumour cell lines (

Table 12). Moreover, the cytotoxicity was also tested against non-tumour cell cultures obtained from porcine liver (data not shown in this section), and all the studied mushrooms showed no significant effect on those cells, at least up to the maximum concentrations tested (400 µg/mL). These results were expected, since the studied mushroom species were carefully identified and used based on their classification as being edible. However, it is important to confirm the lack of effect in hepatocytes of the mushroom's concentrations tested.

Table 12. Antiproliferative effects of the studied edible mushroom species (mean ± SD).

Species	GI_{50} (µg/mL extract)					
	HCT-15	MCF-7	NCI-H460	AGS	HeLa	HepG2
Cultivated						
Cordyceps militaris (L.) Link	73 ± 3^{a}	90.1 ± 0.4^{b}	48 ± 7^{c}	-	66 ± 4	>400
Wild						
Leccinum vulpinum Watling	53 ± 6^{b}	98 ± 10^{a}	114 ± 8^{a}	135 ± 2^{a}	-	-
Ganoderma lucidum (Curtis) P. Karst.	69 ± 2^{a}	67 ± 3^{c}	78 ± 3^{b}	66 ± 6^{b}	-	-

The different accuracy in the expression of the results is explained by the maintenance of the decimal places allowed by the magnitude of standard deviation. (-) not performed. Values correspond to the mean ± SD of three independent experiments, carried out with duplicates. GI_{50} values correspond to the extract concentration causing 50% of growth inhibition in human tumor cell lines.

Different extracts/compounds from several species of mushrooms were tested in human tumour cell lines in order to study their growth inhibitory activity (Heleno et al., 2014; Patel & Goyal, 2012; Vaz et al., 2012a). From the studied species, one of them, the *G. lucidum,* has been reported as a medicinal mushroom with antitumour potential (Calviño et al., 2010; Ferreira et al., 2015; Harhaji Trajković et al., 2009; Liang et al., 2012; Oliveira et al., 2014). Some of these authors have reported GI_{50} values for this species between 93 – 387 mg/mL in the same cell lines as the ones used in this study (Oliveira et al., 2014). However, to the best of our knowledge, this is the first report for *L. vulpinum* bioactivity. Overall, the data obtained highlights the antitumour potential of these mushrooms. Other scientists proved that an aqueous extract from *C. militaris* induced programmed cell death on a human promyelocytic leukemia cell line (Lee et al., 2006) and on human glioblastoma cell lines (GBM8401 and U-87MG), which consisted on apoptosis and autophagy (Yang et al., 2012b). Similar studies have been carried out with several extracts of *G. lucidum*, proving that this species induces programmed cell death, mainly apoptosis, on different human tumour cell lines (Hernandez-Marquez et al., 2014; Hong et al., 2004; Hsieh & Wu, 2013; Hu et al., 2002; Jang et al., 2010; Ji et al., 2011; Jiang et al., 2004; Müller et al., 2006).

Therefore, other studies should be performed based on the published literature, for the evaluation of mushrooms antitumour potential, pharmacological investigation and clinical trials.

In the present work, given the promising results obtained in the screening, the potential mechanism of action of some species (*G. lucidum* and *L. vulpinum*) was elucidated (articles 9 and 10). As reported, the antitumour potential of these species, in different cell lines, was found to be mediated by the induction of programmed cell death, namely apoptosis and autophagy.

As previously referred, the methanolic extract of *L. vulpinum* inhibited the cell growth of different cell lines (HCT-15, NCI-H460, MCF-7 and AGS), with the lowest GI_{50} concentrations having been obtained in the HCT-15 and MCF-7 cell lines. It was decided to continue the studies in the MCF-7 cells since there is evidence for an inverse relationship between mushroom consumption and breast cancer risk (Hong et al., 2008; Roupas et al., 2012; Shin et al., 2010; Zhang et al., 2009). Overall, the extract inhibited the growth of the human MCF-7 breast cancer cell line, by inducing DNA damage, inhibiting cellular proliferation and leading to cell death by apoptosis. This extract also revealed cytotoxic

effects in the human breast non-malignant cell line MCF-10A (used as control), but only when tested at much higher concentrations.

Regarding the *G. lucidum*, previous studies have shown evidence that its methanolic extract affects cellular autophagy. Therefore, AGS cells were treated with this extract, in order to see if it induced autophagy or decreased the autophagic flux. It was found that the extract increased the formation of autophagosomes (vacuoles typical of autophagy). Moreover, the cellular levels of LC3-II were also increased, and the cellular levels of p62 decreased, confirming that the extract affects cellular autophagy. When treating the cells with the extract together with lysossomal protease inhibitors, the cellular levels of LC3-II and p62 increased. These results proved that, in AGS cells, the methanolic extract of *G. lucidum* causes an induction of autophagy, rather than a reduction in the autophagic flux.

Part VII

Conclusions

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Parte VII

Conclusiones

Conclusions

Mushrooms from the Northeastern Portugal proved to have a great nutritional value, being low-calorie foods, rich in polyols, polyunsaturated fatty acids and a source of bioactive compounds (tocopherols, phenolic and organic acids), which may confer antioxidant, antimicrobial and antitumour potential. Therefore, these mushrooms could be considered functional foods and a source of nutraceutical / pharmaceutical products. These data increase knowledge and promote the traditional use and consumption of these natural products.

1) Nutritional value: The studied edible mushrooms revealed low fat contents and quite low energetic values.

The soluble sugars found in all the mushroom species (both cultivated and wild) were mannitol and trehalose, *A. bisporus* and both *Pleurotus* species revealed the highest levels of these sugars. Moreover, the cultivated *F. velutipes* stands out for having the higher contents of fructose (not detected in all the species), and the cultivated *P. ostreatus*, revealed the presence of sucrose.

Regarding the fatty acids profile, in almost all the species unsaturated fatty acids prevailed over saturated fatty acids. The exception was the cultivated *P. linteus*, which stands out as the species with the highest contents of SFA (58.3%). Generally, the highest contents of MUFA were detected in the wild species and the highest PUFA contents in the cultivated species.

2) Bioactive compounds: Wild mushrooms seem to be richer in bioactive compounds; some of them revealed the presence of ascorbic acid, as well as the highest contents of oxalic, malic, quinic and fumaric acids.

Regarding all the phenolic acids identified, as well as the related compound cinnamic acid, these prevailed in the wild species. Nevertheless, the cultivated *A. bisporus* revealed the highest levels of gallic acid.

Regarding the vitamin E isoforms, the highest quantities of α-tocopherol were found in both cultivated species *C. militaris* and *P. linteus*. However, all the other isoforms were mainly detected in the wild species (*i.e.*, β-tocopherol prevailed in both *S. granulatus* and *L. vulpinum*; γ-tocopherol prevailed in *S. luteus, L. vulpinum, C. violaceus* and *A. caesarea*; and δ-tocopherol prevailed in *S. granulatus* from Portugal, *S. luteus*, *A. caesarea* and *L. volemus*).

Regarding their ecology, some of the studied wild species (*i.e., A. caesarea, C. violaceus, L. volemus*, *L. molle, L. vulpinum*, *S. granulatus* and *S. luteus*) are mycorrhizal mushrooms. These species seem to be an exceptional source of bioactive compounds. This may be due to the mycorrhization process itself, since it is related to metabolites production. Although the profile of the investigated metabolites in these species was similar to the profile found in other species, this symbiosis can be further investigated to obtain novel compounds, which were not studied in this work.

3) Bioactive properties: The studied wild species revealed the highest contents of bioactive compounds as also presented greater bioactive potential. This was verified for the antioxidant activity of the species, since both *S. granulatus* and *L. vulpinum* revealed the lower EC_{50} values for all the performed *in vitro* assays.

Regarding the antimicrobial potential, only one cultivated species and one wild species were studied and compared (*C. militaris* and *S. granulatus* from Portugal and Serbia). Once again, the better results were obtained for the wild species.

Regarding the evaluation of the antiproliferative activity, no similar trend was observed when comparing activity with contents in bioactive compounds. However, all studied species (*C. militaris, L. vulpinum* and *G. lucidum*) inhibited the growth of the tested human tumour cell lines.

In addition, by investigating the possible mechanisms of action of the mushrooms extracts (*L. vulpinum* and *G. lucidum*), it was possible to verify that the species under study demonstrated antitumour potential by different mechanisms. *G. lucidum* induced autophagic cell death. Thus, *G. lucidum* and/or its compounds may be studied as a new

source for compounds that induce cancer cell death. Unlike *G. lucidum*, *L. vulpinum* induced apoptosis and caused cellular DNA damage.

The results presented in this thesis indicate that Portuguese wild edible mushrooms may have value as inducers of different types of programmed cell death in human tumour cells, and thus may contain potential antitumour compounds. It would be interesting to attempt the isolation of compounds with antitumour activity (from the extracts which presented tumour cell growth inhibitory potential) and this may be the basis for future work.

Conclusiones

Las setas objeto de estudio, procedentes del Noreste de Portugal, presentaron un gran valor nutricional, siendo alimentos bajos en calorías, ricos en polioles, ácidos grasos poliinsaturados y fuente de compuestos compuestos potencialmente bioactivos (tocoferoles, ácidos fenólicos y orgánicos) que les confieren a dichas especie propiedades antioxidantes, antimicrobianas y antitumorales. Así, las setas pueden considerarse como alimentos funcionales y una fuente de productos nutracéuticos y/o farmacéuticos. Estos datos aumentan el conocimiento y promueven el consumo tradicional de estos produtos naturales.

1) Valor nutricional: Las setas comestibles estudiadas presentaron bajos contenidos de grasa y valores energéticos bastante bajos.

Los azúcares solubles encontrados en todas las especies de setas (tanto cultivadas como silvestres) fueron mannitol y trehalosa, *A. bisporus* y ambas especies de *Pleurotus* destacaron por presentar los niveles más altos de estos azúcares. Mientras que *F. velutipes* destacó por su contenido en fructosa (no detectado en todas las especies), y *P. ostreatus* por la presencia de sacarosa.

Respecto al perfil de los ácidos grasos, en casi todas las especies predominaron los ácidos grasos insaturados frente a los ácidos grasos saturados, excepto en el caso de *P. linteus* (cultivada), que presentó un mayor porcentaje relativo de AGS (58,3%). En general, las especies silvestres destacan por presentar mayores porcentajes de AGMI y las cultivadas por los AGPI.

2) Compuestos bioactivos: Las setas silvestres parecen ser más ricas en compuestos bioactivos que las cultivadas. En algunas especies silvestres se observaron niveles interesantes de ácido ascórbico, así como los contenidos más altos de otros ácidos orgánicos como el ácidos oxálico, málico, quínico y fumárico.

Las especies silvestres descaron por su contenido en todos los ácidos fenólicos identificados, así como por el ácido cinámico. En *A. bisporus* (cultivado) se observó la mayor concentración de ácido gálico.

En cuanto a las isoformas de vitamina E, se encontraron las mayores cantidades de α-tocoferol en ambas especies cultivadas *C. militaris* y *P. linteus*. El resto de isoformas se detectaron principalmente en las especies silvestres (es decir, β-tocoferol prevaleció tanto en *S. granulatus* y *L. vulpinum*, γ-tocoferol prevaleció en *S. luteus*, *L. vulpinum*, *C. violaceus* y *A. caesarea* y δ-tocoferol prevaleció en *S. granulatus* de Portugal, *S. luteus*, *A. caesarea* y *L. volemus*).

Algunas de las especies silvestres estudiadas (*A. caesarea*, *C. violaceus*, *L. volemus*, *L. molle*, *L. vulpinum*, *S. granulatus* y *S. luteus*), son hongos micorrízicos. Estas especies parecen ser una fuente excepcional de compuestos bioactivos. Esto puede deberse al propio proceso de micorrización, ya que está relacionado con la producción de metabolitos. Aunque el perfil de los metabolitos evaluados en estas especies fue similar al de los otros, esta simbiosis podría utilizarse para obtener nuevos compuestos, los cuales no fueron encuesta en este trabajo.

3) Actividad biológica: Las especies con los mayores contenidos de compuestos bioactivos también presentaron la mayor actividad biológica en terminos de capacidad antioxidante, antimicrobiana y antitumoral, siendo especialmente patente en las setas silvestres estudiadas.

Como ocurrió para la actividad antioxidante, en el caso de *S. granulatus* y *L. vulpinum*, las cuales presentaron los menores valores de EC_{50} para todos los ensayos *in vitro* realizados y por lo tanto mayor capacidad antioxidante.

En cuanto al potencial antimicrobiano, sólo se estudiaron y compararon una sola especie cultivada y una especie silvestre (*C. militaris* y *S. granulatus* de Portugal y Serbia). Una vez más, los mejores resultados se obtuvieron en las especies silvestres.

Para la evaluación de la actividad antiproliferativa, se ha demostrado que todas las especies estudiadas (*C. militaris, L. vulpinum* y *G. lucidum*) inhiben el crecimiento celular de las líneas celulares tumorales humanas ensayadas.

Además, al investigar los posibles mecanismos de acción de los extractos de las setas, *L. vulpinum* y *G. lucidum* demostraron potencial antitumoral aparentemente a través de diferentes mecanismos. Los resultados obtenidos demuestran que *G. lucidum* es un inductor de la autofagia, de modo que *G. lucidum* y/o sus compuestos pueden ser explotados como un nuevo enfoque para terapias basadas en la inducción de la muerte de células cancerosas. Mientras que *L. vulpinum* resultó ser un inductor de la apoptosis, así como causar daños en el ADN celular.

Los resultados obtenidos en esta memoria, indican que las setas comestibles silvestres portuguesas pueden tener valor como inductores de diferentes tipos de muerte celular programada en líneas celulares tumorales humanas. Sería interesante intentar el aislamiento de los compuestos con actividad antitumoral (de los extractos que presentaban potencial inhibidor del crecimiento de células tumorales) y esto puede ser la base para el futuros trabajos (nuevos hallazgos de potenciales agentes antitumorales).

Part VIII

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ANNEX 1. SUPPLEMENTARY MATERIAL

Table S1. Vitamin C and main organic acids found in Portuguese edible mushrooms.

Mushroom species	Ascorbic acid Other organic acids	Quantity	Reference
Agaricus albertii (Bon.)	Oxalic acid	0.62 [a]	Reis et al., 2014b
	Quinic acid	0.65 [a]	
	Malic acid	1.39 [a]	
	Citric acid	0.71 [a]	
	Fumaric acid	0.16 [a]	
Agaricus arvensis Schaeff.	Ascorbic acid	0.002 – 0.04 [b]	Barros et al., 2007a and 2008a
Agaricus bisporus (J.E. Lange) Imbach	Ascorbic acid	0.003 [b]	Barros et al., 2008a and 2008b
	Oxalic acid	1.53 – 1.96 [a]	Barros et al., 2013
	Quinic acid	0.64 [a]	
	Malic acid	2.95 – 3.01 [a]	
	Citric acid	3.46 – 4.32 [a]	
	Fumaric acid	0.11 – 0.26 [a]	
Agaricus campestris L. ex Fr.	Ascorbic acid	0.02 [a]	Pereira et al., 2012
	Oxalic acid	1.13 [a]	Barros et al., 2013
	Malic acid	1.78 [a]	
	Fumaric acid	0.30 [a]	
Agaricus comtulus Fr.	Oxalic acid	0.96 [a]	Barros et al., 2013
	Quinic acid	0.79 [a]	
	Malic acid	1.13 [a]	
	Citric acid	2.66 [a]	
	Fumaric acid	0.20 [a]	
Agaricus lutosus (F.H. Møller) F.H. Møller	Ascorbic acid	0.03 [a]	Pereira et al., 2012
	Oxalic acid	0.59 [a]	Barros et al., 2013
	Malic acid	1.16 [a]	
	Citric acid	5.83 [a]	
	Fumaric acid	0.35 [a]	
Agaricus silvaticus Schaeff ex. Secr.	Ascorbic acid	0.004 [b]	Barros et al., 2008a and 2008b
	Oxalic acid	0.49 [a]	Barros et al., 2013
	Malic acid	2.39 [a]	
	Citric acid	4.30 [a]	
	Fumaric acid	0.38 [a]	
Agaricus silvicola (Vittad.) Peck	Ascorbic acid	0.004 [b]	Barros et al., 2008a and 2008b

Agaricus urinascens var. *excellens* (F.H. Møller) Nauta	Oxalic acid	0.87 [a]	Reis et al., 2014b
	Malic acid	0.76 [a]	
	Citric acid	0.50 [a]	
	Fumaric acid	0.10 [a]	
Amanita caesarea (Scop.) Pers	Ascorbic acid	0.18 – 0.21 [a]	Reis et al., 2011b Valentão et al., 2005a
	Oxalic acid	0.17 – 0.35 [a]	Barros et al., 2013 Fernandes et al., 2015 Valentão et al., 2005a
	Citric acid + Ketoglutaric acid	0.05 [a]	
	Malic acid	1.10 – 1.62 [a]	
	Succinic acid	0.000007 [a]	
	Shikimic acid	0.0002 [a]	
	Fumaric acid	0.004 – 0.50 [a]	
Amanita crocea (Quél. in Bourd.) Singer ex Singer	Oxalic acid	0.28 [a]	Leal et al., 2013
	Quinic acid	0.23 [a]	
	Malic acid	0.94 [a]	
	Citric acid	2.07 [a]	
	Fumaric acid	0.33 [a]	
Amanita curtipes E.-J. Gilbert	Oxalic acid	0.27 [a]	Fernandes et al., 2015
	Malic acid	1.50 [a]	
	Fumaric acid	0.26 [a]	
Amanita mairei (Foley)	Oxalic acid	0.26 [a]	Leal et al., 2013
	Citric acid	2.63 [a]	
	Fumaric acid	0.30 [a]	
Amanita rubescens (Pers. ex Fr.) Gray	Ascorbic acid	0.34 [a]	Ribeiro et al., 2006
	Oxalic acid	0.06 – 0.19 [a]	Ribeiro et al., 2006 and 2008b
	Quinic acid	6.31 – 12.41 [a]	
	Malic acid	0.77 – 1.49 [a]	
	Citric acid	0.13 – 1.94 [a]	
	Aconitic acid	0.28 [a]	
	Ketoglutaric acid	0.10 – 5.02 [a]	
	Succinic acid	0.002 – 0.03 [a]	
	Shikimic acid	0.002 – 0.01 [a]	
	Fumaric acid	0.17 – 2.10 [a]	
Amanita spissa (Fr.) Kummer	Oxalic acid	tr (< 0.007) [a]	Barros et al., 2013
	Malic acid	2.62 [a]	
	Citric acid	1.89 [a]	
	Fumaric acid	0.51 [a]	
Armillaria mellea (Vahl. ex Fr.) Kummer	Ascorbic acid	0.15 [a]	Vaz et al., 2011a
	Oxalic acid	0.14 [a]	Barros et al., 2013
	Quinic acid	0.82 [a]	
	Malic acid	1.38 [a]	
	Fumaric acid	0.27 [a]	
Boletus aereus Bull.	Oxalic acid	2.08 [a]	Barros et al., 2013
	Malic acid	8.57 [a]	
	Fumaric acid	0.03 [a]	
Boletus armeniacus Quél.	Oxalic acid	6.22 [a]	Barros et al., 2013

	Malic acid	11.80 [a]	
	Fumaric acid	0.06 [a]	
Boletus citrinoporus Halling	Oxalic acid	0.56 [a]	Barros et al., 2013
	Malic acid	0.83 [a]	
	Fumaric acid	0.13 [a]	
Boletus edulis Bull.	Oxalic acid	0.01 – 2.26 [a]	Barros et al., 2013 Fernandes et al., 2013a Ribeiro et al., 2006 and 2008b Valentão et al., 2005a
	Malic acid	0.79 – 1.73 [a]	
	Citric acid	0.05– 4.7 [a]	
	Aconitic acid	0.21 – 0.44 [a]	
	Succinic acid	0.000004 – 0.02 [a]	
	Fumaric acid	0.001 – 0.22 [a]	
Boletus fragrans Vittad.	Oxalic acid	0.19 [a]	Barros et al., 2013
	Quinic acid	2.30 [a]	
	Malic acid	1.71 [a]	
	Citric acid	3.06 [a]	
	Fumaric acid	0.09 [a]	
Boletus impolitus Fr.	Ascorbic acid	0.002 [a]	Pereira et al., 2012
	Oxalic acid	0.44 [a]	Barros et al., 2013
	Malic acid	0.76 [a]	
	Fumaric acid	0.24 [a]	
Boletus porosporus Imler ex Bon & G. Moreno	Oxalic acid	0.34 [a]	Leal et al., 2013
	Quinic acid	1.93 [a]	
	Citric acid	0.21 [a]	
	Fumaric acid	0.07 [a]	
Boletus regius Krombh.	Oxalic acid	0.17 [a]	Leal et al., 2013
	Quinic acid	0.18 [a]	
	Citric acid	3.32 [a]	
	Fumaric acid	0.07 [a]	
Boletus reticulatus Schaeff.	Oxalic acid	0.39 [a]	Barros et al., 2013
	Malic acid	0.46 [a]	
	Fumaric acid	0.03 [a]	
Bovista aestivalis (Bonord.) Demoulin	Oxalic acid	1.06 [a]	Barros et al., 2013
	Malic acid	tr (< 0.007) [a]	
	Fumaric acid	0.01 [a]	
Bovista nigrescens (Pers.)	Oxalic acid	0.08 [a]	Barros et al., 2013
	Malic acid	0.05 [a]	
	Fumaric acid	tr (< 0.007) [a]	
Calocybe gambosa (Fr.) Donk	Ascorbic acid	0.04 [b]	Barros et al., 2008b Vaz et al., 2011a
		0.18 [a]	
	Oxalic acid	1.19 [a]	Barros et al., 2013
	Malic acid	2.44 [a]	
	Fumaric acid	0.05 [a]	
Calvatia utriformis (Bull.) Jaap.	Ascorbic acid	0.15 [a]	Grangeia et al., 2011
Cantharellus cibarius Fr.	Ascorbic acid	0.04 – 0.09 [b]	Barros et al., 2008b and 2008c Valentão et al., 2005b
		0.002 – 0.02 [a]	

	Oxalic acid	0.13 – 0.29 [a]	Barros et al., 2013 Valentão et al., 2005b
	Malic acid	0.02 – 5.94 [a]	
	Citric acid	0.003 – 1.20 [a]	
	Shikimic acid	tr (≤ 0.00009) [a]	
	Fumaric acid	0.00004 – 0.25 [a]	
Chlorophyllum rhacodes (Vittadini) Vellinga	Oxalic acid	1.02 [a]	Barros et al., 2013
	Malic acid	0.56 [a]	
	Citric acid	3.47 [a]	
	Fumaric acid	0.63 [a]	
Clavariadelphus pistillaris (L.) Donk	Ascorbic acid	0.003 [a]	Pereira et al., 2012
	Oxalic acid	0.10 [a]	Barros et al., 2013
	Malic acid	2.12 [a]	
	Fumaric acid	0.91 [a]	
Clavariadelphus truncatus (Quél.) Donk	Ascorbic acid	0.007 [a]	Pereira et al., 2012
	Oxalic acid	0.39 [a]	Barros et al., 2013
	Malic acid	0.27 [a]	
	Citric acid	0.78 [a]	
	Fumaric acid	0.12 [a]	
Clitocybe costata Kühner & Romagn.	Oxalic acid	0.81 [a]	Barros et al., 2013
	Malic acid	2.49 [a]	
	Citric acid	2.67 [a]	
	Fumaric acid	0.33 [a]	
Clitocybe gibba (Pers.) P. Kumm.	Ascorbic acid	0.02 [a]	Pereira et al., 2012
	Oxalic acid	1.26 [a]	Barros et al., 2013
	Malic acid	0.33 [a]	
	Fumaric acid	0.33 [a]	
Clitocybe odora (Fr.) P. Kumm.	Ascorbic acid	0.17 [a]	Vaz et al., 2011a
	Oxalic acid	1.41 [a]	Barros et al., 2013
	Quinic acid	19.82 [a]	
	Malic acid	0.43 [a]	
	Fumaric acid	0.12 [a]	
Clitocybe subconnexa Murrill	Oxalic acid	2.64 [a]	Heleno et al., 2015a
	Fumaric acid	0.23 [a]	
Clitopilus prunulus (Scop. ex Fr.) P. Kumm	Ascorbic acid	0.40 [a]	Grangeia et al., 2011
Coprinus comatus (O.F.Müll.) Pers.	Ascorbic acid	0.13 [a]	Vaz et al., 2011a
	Oxalic acid	0.49 [a]	Barros et al., 2013
	Malic acid	2.03 [a]	
	Fumaric acid	0.85 [a]	
Cortinarius praestans Cordier	Ascorbic acid	0.009 [a]	Pereira et al., 2012
	Oxalic acid	0.15 [a]	Barros et al., 2013
	Malic acid	1.93 [a]	
	Citric acid	1.34 [a]	
	Fumaric acid	1.23 [a]	
Cortinarius violaceus (L.: Fr.) Gray	Ascorbic acid	0.07 [a]	Reis et al., 2011b
	Oxalic acid	0.18 [a]	Barros et al., 2013

	Quinic acid	0.40 [a]	
	Malic acid	0.87 [a]	
	Citric acid	0.53 [a]	
	Fumaric acid	0.87 [a]	
Craterellus cornucopioides (L.) Pers.	Ascorbic acid	0.09 [b]	Barros et al., 2008b
	Oxalic acid	0.33 [a]	Barros et al., 2013
	Malic acid	2.78 [a]	
	Fumaric acid	0.26 [a]	
Fistulina hepatica (Schaeff.) With.	Ascorbic acid	0.01 – 0.28 [a]	Ribeiro et al., 2007
	Oxalic acid	0.0005 – 0.02 [a]	Barros et al., 2013 Ribeiro et al., 2007
	Malic acid	0.02 – 3.34 [a]	
	Citric acid	0.003 – 2.97 [a]	
	Aconitic acid	0.0007 – 0.008 [a]	
	Fumaric acid	0.003 – 0.38 [a]	
Flammulina velutipes (Curtis) Singer	Ascorbic acid	0.02 [a]	Pereira et al., 2012
	Oxalic acid	0.51 – 1.41 [a]	Barros et al., 2013
	Malic acid	1.85 – 3.28 [a]	
	Citric acid	6.05 [a]	
	Fumaric acid	0.16 – 0.21 [a]	
Gyromitra esculenta (Pers. ex Pers.) Fr.	Oxalic acid	0.13 [a]	Leal et al., 2013
	Quinic acid	1.43 [a]	
	Malic acid	0.69 [a]	
	Citric acid	1.46 [a]	
	Fumaric acid	0.36 [a]	
Gyroporus castaneus (Bull.) Quel.	Citric acid + Ketoglutaric acid	0.12 [a]	Valentão et al., 2005a
	Malic acid + Quinic acid	0.38 [a]	
	Succinic acid	0.00002 [a]	
	Fumaric acid	0.007 [a]	
Helvella lacunosa (Afzel.)	Oxalic acid	2.98 [a]	Leal et al., 2013
	Quinic acid	0.24 [a]	
	Malic acid	1.96 [a]	
	Citric acid	1.42 [a]	
	Fumaric acid	0.33 [a]	
Hericium erinaceus (Bull.) Persoon	Oxalic acid	0.06 [a]	Heleno et al., 2015b
	Malic acid	3.09 [a]	
	Fumaric acid	0.60 [a]	
Hericium coralloides (Scop.) Pers.	Oxalic acid	0.09 [a]	Heleno et al., 2015b
	Malic acid	0.47 [a]	
	Fumaric acid	0.37 [a]	
Hydnum repandum L.	Oxalic acid	0.003 [a]	Fernandes et al., 2013a
	Malic acid	3.47 [a]	
	Citric acid	3.82 [a]	
	Fumaric acid	0.61 [a]	
Hygrophoropsis aurantiaca (Wulfen) Maire	Oxalic acid	0.52 [a]	Barros et al., 2013
	Malic acid	1.46 [a]	
	Fumaric acid	0.10 [a]	

Hygrophorus agathosmus (Fr.) Fr.	Oxalic acid	0.16 – 0.22 [a]	Ribeiro et al., 2006
	Quinic acid + Malic acid	2.35 – 2.77 [a]	
	Citric acid	2.05 – 2.29 [a]	
	Aconitic acid	0.18 [a]	
	Ketoglutaric acid	0.24 – 0.26 [a]	
	Succinic acid	0.44 – 0.85 [a]	
	Shikimic acid	0.01 [a]	
	Fumaric acid	0.62 – 2.10 [a]	
Hygrophorus chrysodon (Fr.) Fr.	Ascorbic acid	0.03 [a]	Pereira et al., 2012
	Oxalic acid	0.49 [a]	Barros et al., 2013
	Malic acid	0.07 [a]	
	Fumaric acid	0.02 [a]	
Hygrophorus pustulatus (Persoon: Fries) Fries	Ascorbic acid	0.39 [a]	Grangeia et al., 2011
Laccaria amethystine (Huds.) Cooke	Oxalic acid	0.20 [a]	Barros et al., 2013
	Malic acid	0.80 [a]	
	Citric acid	1.43 [a]	
	Fumaric acid	0.66 [a]	
Lactarius deliciosus (L. ex Fr.) S.F.Gray	Ascorbic acid	0.008 – 0.10 [a]	Barros et al., 2007b Valentão et al., 2005a
	Oxalic acid	0.0005 – 0.51 [a]	Barros et al., 2013 Valentão et al., 2005a
	Malic acid	2.33 [a]	
	Citric acid + ketoglutaric acid	0.009 – 0.61 [a]	
	Succinic acid	tr (< 0.0001) [a]	
	Fumaric acid	0.001 – 0.11 [a]	
Lactarius piperatus (L.) Pers.	Ascorbic acid	0.003 – 0.02 [b]	Barros et al., 2007b
Lactarius volemus (Fr.) Fr.	Ascorbic acid	0.08 [a]	Reis et al., 2011b
	Oxalic acid	0.66 [a]	Barros et al., 2013
	Quinic acid	0.12 [a]	
	Malic acid	2.98 [a]	
	Fumaric acid	0.25 [a]	
Lentinula edodes (Berk.) Pegler	Oxalic acid	1.01 [a]	Barros et al., 2013
	Malic acid	2.89 [a]	
	Citric acid	16.56 [a]	
	Fumaric acid	0.50 [a]	
Lepista nuda (Bull.) H.E. Bigelow & A.H. Sm.	Ascorbic acid	0.023 [b]	Barros et al., 2008c
	Oxalic acid	4.34 [a]	Barros et al., 2013
	Quinic acid	12.53 [a]	
	Malic acid	0.87 [a]	
	Fumaric acid	0.07 [a]	
Leucoagaricus leucothites (Vittad.) Wasser	Ascorbic acid	0.02 [a]	Pereira et al., 2012
	Oxalic acid	0.33 [a]	Barros et al., 2013
	Malic acid	1.74 [a]	
	Fumaric acid	0.59 [a]	
Leucopaxillus giganteus (Sowerby) Singer	Ascorbic acid	0.01 [b]	Barros et al., 2007a
	Oxalic acid	0.21 [a]	Barros et al., 2013

	Malic acid	6.03 [a]	
	Fumaric acid	0.23 [a]	
Lycoperdon echinatum Pers.	Ascorbic acid	0.17 [a]	Grangeia et al., 2011
Lycoperdon molle Pers.	Ascorbic acid	0.03 [b]	Barros et al., 2008c
Lycoperdon perlatum Pers.	Ascorbic acid	0.02 [b]	Barros et al., 2008c
Lycoperdon umbrinum Pers.	Oxalic acid	0.14 [a]	Barros et al., 2013
	Malic acid	tr (< 0.007) [a]	
	Fumaric acid	0.02 [a]	
Lyophyllum decastes (Fries: Fries) Singer	Ascorbic acid	0.39 [a]	Grangeia et al., 2011
Macrolepiota excoriata (Schaeff.) Wasser	Ascorbic acid	0.18 [a]	Grangeia et al., 2011
	Oxalic acid	0.64 [a]	Barros et al., 2013
	Malic acid	2.37 [a]	
	Fumaric acid	0.24 [a]	
Macrolepiota procera (Scop.) Singer	Oxalic acid	1.33 [a]	Barros et al., 2013
	Malic acid	0.97 [a]	
	Citric acid	2.64 [a]	
	Fumaric acid	0.04 [a]	
Marasmius oreades (Bolton) Fr	Oxalic acid	1.80 [a]	Barros et al., 2013
	Malic acid	7.86 [a]	
	Citric acid	4.36 [a]	
	Fumaric acid	0.04 [a]	
Phellinus linteus (Berkeley & Curtis) Teng	Oxalic acid	0.30 [a]	Reis et al., 2014c
Pleurotus eryngii (DC.) Quél.	Oxalic acid	0.20 – 0.25 [a]	Barros et al., 2013 Reis et al., 2014b
	Malic acid	1.85 – 6.15 [a]	
	Citric acid	0.43 – 2.87 [a]	
	Fumaric acid	0.25 – 0.55 [a]	
Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm.	Oxalic acid	0.44 [a]	Barros et al., 2013
	Malic acid	1.51 [a]	
	Citric acid	2.14 [a]	
	Fumaric acid	0.34 [a]	
Ramaria aurea (Schaeff.) Quél.	Ascorbic acid	0.0007 [a]	Pereira et al., 2012
	Oxalic acid	0.14 [a]	Barros et al., 2013
	Malic acid	0.46 [a]	
	Citric acid	0.44 [a]	
	Fumaric acid	0.48 [a]	
Russula aurea Pers.	Oxalic acid	1.09 [a]	Leal et al., 2013
	Malic acid	4.53 [a]	
	Citric acid	1.20 [a]	
	Fumaric acid	0.38 [a]	
Russula cyanoxantha (Schaeff.) Fr.	Ascorbic acid	0.19 [a]	Grangeia et al., 2011
	Oxalic acid	0.05 – 0.12 [a]	Ribeiro et al., 2006 and 2008b
	Quinic acid	3.52 – 6.38 [a]	
	Malic acid	3.16 – 5.86 [a]	
	Citric acid	0.17 – 0.85 [a]	

	Aconitic acid	0.01 – 0.04 [a]	
	Succinic acid	0.09 – 0.23 [a]	
	Fumaric acid	0.53 – 0.83 [a]	
Russula delica Fr.	Oxalic acid	1.01 [a]	Barros et al., 2013
	Malic acid	2.95 [a]	
	Fumaric acid	0.23 [a]	
Russula olivacea (Schaeff.) Fr.	Ascorbic acid	0.25 [a]	Grangeia et al., 2011
	Oxalic acid	0.37 [a]	Barros et al., 2013
	Malic acid	1.17 [a]	
	Fumaric acid	0.22 [a]	
Russula virescens (Schaeff.) Fr.	Oxalic acid	0.78 [a]	Leal et al., 2013
	Malic acid	2.71 [a]	
	Citric acid	0.55 [a]	
	Fumaric acid	0.23 [a]	
Sarcodon imbricatus (L.) P. Karst.	Ascorbic acid	0.02 [b]	Barros et al., 2007a
	Oxalic acid	1.27 [a]	Barros et al., 2013
	Malic acid	24.07 [a]	
	Fumaric acid	0.08 [a]	
Suillus bellinii (Inzenga) Watling	Oxalic acid	0.16 – 0.33 [a]	Ribeiro et al., 2006
	Quinic acid + Malic	0.91 – 3.12 [a]	
	Citric acid	0.62 – 1.12 [a]	
	Succinic acid	0.57 – 0.69 [a]	
	Shikimic acid	0.01 [a]	
	Fumaric acid	0.53 – 0.75 [a]	
Suillus collinitus (Fr.) Kuntze	Ascorbic acid	0.009 – 0.38 [a]	Valentão et al., 2005a
	Oxalic acid	0.003 – 0.01 [a]	Valentão et al., 2005a
	Citric acid + Ketoglutaric acid	0.04 – 0.29 [a]	
	Malic acid + Quinic acid	0.05 – 0.27 [a]	
	Succinic acid	tr (< 0.0009) [a]	
	Fumaric acid	0.005 – 0.04 [a]	
Suillus granulatus (L.) Roussel	Oxalic acid	0.02 – 3.35 [a]	Reis et al., 2014a Ribeiro et al., 2006 and 2008b
	Quinic acid	0.29 – 8.64 [a]	
	Malic acid	0.09 – 1.37 [a]	
	Citric acid	0.14 – 4.79 [a]	
	Aconitic acid	0.15 – 5.27 [a]	
	Succinic acid	0.007 – 0.22 [a]	
	Shikimic acid	0.01 – 0.03 [a]	
	Fumaric acid	0.14 – 2.82 [a]	
Suillus luteus (L.: Fries) Gray	Ascorbic acid	0.09 [a]	Reis et al., 2011
	Oxalic acid	0.20 – 0.69 [a]	Ribeiro et al., 2006
	Quinic acid + Malic acid	1.66 – 4.50 [a]	
	Citric acid	0.38 – 1.03 [a]	
	Aconitic acid	0.97 [a]	
	Succinic acid	0.13 – 0.23 [a]	
	Shikimic acid	0.01 [a]	
	Fumaric acid	0.87 – 1.38 [a]	

Suillus variegatus (Sw.) Kuntze	Ascorbic acid	0.006 [a]	Pereira et al., 2012
	Oxalic acid	2.46 [a]	Barros et al., 2013
	Malic acid	0.38 [a]	
	Fumaric acid	0.02 [a]	
Tricholoma acerbum (Bull.) Quél.	Ascorbic acid	0.02 [b]	Barros et al., 2008c
Tricholoma imbricatum (Fr.) P.Kumm.	Oxalic acid	0.33 [a]	Barros et al., 2013
	Malic acid	4.43 [a]	
	Fumaric acid	0.63 [a]	
Tricholoma portentosum (Fr.) Quél.	Oxalic acid	0.43 [a]	Barros et al., 2013
	Malic acid	6.49 [a]	
	Citric acid	1.90 [a]	
	Fumaric acid	0.50 [a]	
Tricholomopsis rutilans (Schaeff.: Fr.) Sing.	Oxalic acid	0.08 – 0.29 [a]	Ribeiro et al., 2006
	Quinic acid + Malic acid	1.69 – 2.41 [a]	
	Citric acid	0.69 – 0.78 [a]	
	Aconitic acid	0.24 [a]	
	Succinic acid	0.84 [a]	
	Shikimic acid	0.01 [a]	
	Fumaric acid	0.37 – 0.49 [a]	
Volvopluteus gloiocephalus (DC.) Vizzini, Contu & Justo	Oxalic acid	0.37 [a]	Heleno et al., 2015a
	Fumaric acid	0.13 [a]	
Xerocomus chrysenteron (Bull.) Šutara	Citric acid + Ketoglutaric acid	0.04 [a]	Valentão et al., 2005a
	Malic acid + Quinic acid	0.30 [a]	
	Succinic acid	0.002 [a]	
	Fumaric acid	0.001 [a]	

tr- trace; values are presented in: [a]g/100 g of dry weight and [b]g/100 g of extract.

Table S2. Main phenolic acids and related compounds found in Portuguese edible mushrooms.

Mushroom species	**Phenolic acid / related compound**	**Quantity**	**Reference**
Agaricus albertii (Bon.)	*p*-Hydroxybenzoic acid	8.23	Reis et al., 2014b
	p-Coumaric acid	3.53	
	Cinnamic acid	2.51	
Agaricus arvensis Schaeff.	*p*-Hydroxybenzoic acid	7.01	Barros et al., 2009
	p-Coumaric acid	4.87	
Agaricus bisporus (J.E. Lange)	Gallic acid	6.28	Barros et al., 2009

Imbach	*p*-Hydroxybenzoic acid	2.56	Reis et al., 2012b
	p-Coumaric acid	0.23	
	Cinnamic acid	0.01 – 0.04	
Agaricus silvicola (Vittad.) Peck	*p*-Hydroxybenzoic acid	23.87	Barros et al., 2009
	p-Coumaric acid	4.57	
Agaricus urinascens var. *excellens* (F.H. Møller) Nauta	*p*-Hydroxybenzoic acid	3.27	Reis et al., 2014b
	p-Coumaric acid	1.33	
	Cinnamic acid	1.75	
Amanita caesarea (Scop.) Pers	Protocatechuic acid	0.67	Reis et al., 2011b Fernandes et al., 2015
	p-Hydroxybenzoic acid	0.72 – 4.7	
	Cinnamic acid	0.03 – 2.48	
Amanita crocea (Quél. in Bourd.) Singer ex Singer	Protocatechuic acid	21.33	Leal et al., 2013
	p-Hydroxybenzoic acid	0.74	
	Cinnamic acid	tr (< 0.13)	
Amanita curtipes E.-J. Gilbert	*p*-Hydroxybenzoic acid	0.10 – 0.32	Fernandes et al., 2015
Amanita mairei (Foley)	*p*-Hydroxybenzoic acid	5.94	Leal et al., 2013
	Cinnamic acid	6.87	
Amanita rubescens (Pers. ex Fr.) Gray	*p*-Hydroxybenzoic acid	49.09	Ribeiro et al., 2006
Armillaria mellea (Vahl. ex Fr.) Kummer	*p*-Hydroxybenzoic acid	0.40	Vaz et al., 2011a
	Cinnamic acid	0.87	
Boletus aereus Bull.	*p*-Hydroxybenzoic acid	1.34	Heleno et al., 2011
	p-Coumaric aci	0.45	
	Cinnamic acid	3.16	
Boletus edulis Bull.	Protocatechuic acid	0.20	Fernandes et al., 2014 Heleno et al., 2011
	p-Hydroxybenzoic acid	0.66	
	p-Coumaric acid	0.12 – 0.56	
	Cinnamic acid	0.37 – 1.11	
Boletus porosporus Imler ex Bon & G. Moreno	*p*-Hydroxybenzoic acid	0.31	Leal et al., 2013
	Cinnamic acid	tr (< 0.13)	
Boletus regius Krombh.	Protocatechuic acid	1.15	Leal et al., 2013
	p-Hydroxybenzoic acid	1.77	
	p-Coumaric acid	2.08	
	Chrysin derivatives	7.33 – 11.16	
	Cinnamic acid	6.05	
Boletus reticulatus Schaeff.	*p*-Hydroxybenzoic acid	1.21	Heleno et al., 2011
	Cinnamic acid	0.28	
Calocybe gambosa (Fr.) Donk	Protocatechuic acid	0.26	Vaz et al., 2011a
	p-Hydroxybenzoic acid	3.84	
	p-Coumaric acid	0.40	
	Cinnamic acid	1.77	
Cantharellus cibarius Fr.	Hydroxytyrosol	21.09 – 43.74	Valentão et al., 2005b
	3-*O*-Caffeoylquinic acid	0.23 – 2.00	
	Tyrosol	3.54 – 45.01	
	4-*O*-Caffeoylquinic acid	0.04 – 0.26	

	5-*O*-Caffeoylquinic acid	0.07 – 0.27	
	Caffeic acid	0.03 – 0.42	
	p-Coumaric acid	0.03 – 0.32	
	Rutin	0.04 – 1.23	
	Luteolin	7.58 – 8.84	
	Apigenin	1.44 – 1.54	
Clitocybe odora (Fr.) P. Kumm.	*p*-Hydroxybenzoic acid	2.79	Vaz et al., 2011a
	p-Coumaric acid	0.18	
	Cinnamic acid	1.38	
Clitocybe subconnexa Murrill	Gallic acid	0.01 – 0.06	Heleno et al., 2015a
	Protocatechuic acid	0.03 – 0.18	
	p-Coumaric acid	0.02 – 016	
	Cinnamic acid	0.01 – 0.07	
Coprinopsis atramentaria (Bull.) Redhead, Vilgalys & Moncalvo	*p*-Hydroxybenzoic acid	4.71	Heleno et al., 2012a
	p-Coumaric acid	0.82	
	Cinnamic acid	1.70	
Coprinus comatus (O.F.Müll.) Pers.	*p*-Hydroxybenzoic acid	6.15	Vaz et al., 2011a
	p-Coumaric acid	1.88	
	Cinnamic acid	1.26	
Cortinarius violaceus (L.: Fr.) Gray	*p*-Hydroxybenzoic acid	1.30	Reis et al., 2011b
	Cinnamic acid	0.56	
Fistulina hepatica (Schaeff.) With.	Protocatechuic acid	6.76	Vaz et al., 2011b Ribeiro et al., 2007
	p-Hydroxybenzoic acid	4.19	
	p-Coumaric acid	9.48 – 16.23	
	Cinnamic acid	0.22	
	Caffeic acid	9.90 – 13.97	
	Ellagic acid	17.11 – 28.46	
Ganoderma lucidum (Curtis) P. Karst.	*p*-Hydroxybenzoic acid	0.58	Heleno et al., 2012b
	p-Coumaric acid	0.38	
	Cinnamic acid	0.28	
Gyromitra esculenta (Pers. ex Pers.) Fr.	Protocatechuic acid	3.74	Leal et al., 2013
	Cinnamic acid	tr (< 0.13)	
Helvella lacunosa (Afzel.)	Protocatechuic acid	0.77	Leal et al., 2013
	p-Hydroxybenzoic acid	0.13	
	p-Coumaric acid	0.17	
Hericium erinaceus (Bull.) Persoon	Gallic acid	0.008 – 0.08	Heleno et al., 2015b
	p-Hydroxybenzoic acid	0.001 – 0.07	
	p-Coumaric acid	0.02 – 0.14	
Hericium coralloides (Scop.) Pers.	Gallic acid	0.01 – 0.13	Heleno et al., 2015b
	p-Hydroxybenzoic acid	0.002 – 0.10	
	p-Coumaric acid	0.02 – 0.15	
Hydnum repandum L.	Cinnamic acid	0.45	Vaz et al., 2011b
Hygrophoropsis aurantiaca (Wulfen) Maire	Cinnamic acid	0.35	Vaz et al., 2011b
Hygrophorus agathosmus (Fr.) Fr.	Protocatechuic acid	1.79	Vaz et al., 2011b

	p-Coumaric acid	0.87	
	Cinnamic acid	4.60	
Hygrophorus olivaceoalbus Fr. (Fr.)	*p*-Hydroxybenzoic acid	0.74	Vaz et al., 2011b
	Cinnamic acid	0.09	
Lactarius deliciosus (L. ex Fr.) S.F.Gray	*p*-Hydroxybenzoic acid	2.27	Barros et al., 2009
Lactarius salmonicolor R.Heim & Leclair	*p*-Hydroxybenzoic acid	0.34	Vaz et al., 2011b
	Cinnamic acid	0.26	
Lactarius volemus (Fr.) Fr.	*p*-Hydroxybenzoic acid	0.46	Reis et al., 2011b
	Cinnamic acid	0.32	
Lentinula edodes (Berk.) Pegler	Protocatechuic acid	0.04	Reis et al., 2012b
	p-Hydroxybenzoic acid	0.16	
	Cinnamic acid	0.002	
Lepista nuda (Bull.) H.E. Bigelow & A.H. Sm.	Protocatechuic acid	3.35	Barros et al., 2009
	p-Hydroxybenzoic acid	2.93	
	p-Coumaric acid	0.38	
Lycoperdon molle Pers.	*p*-Hydroxybenzoic acid	4.17	Barros et al., 2009
	Vanillic acid isomers	0.40 – 3.60	
Pleurotus eryngii (DC.) Quél.	Protocatechuic acid	0.01	Reis et al., 2012b and 2014b
	p-Hydroxybenzoic acid	0.01 – 3.81	
	p-Coumaric acid	0.10	
	Cinnamic acid	0.02 – 0.85	
Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm.	Protocatechuic acid	0.08	Reis et al., 2012b
	p-Hydroxybenzoic acid	0.16	
	p-Coumaric acid	0.08	
	Cinnamic acid	0.02	
Russula aurea Pers.	*p*-Hydroxybenzoic acid	1.02	Leal et al., 2013
	Cinnamic acid	17.15	
Russula caerulea (Pers.) Fr.	Cinnamic acid	0.26	Vaz et al., 2011b
Russula delica Fr.	Gallic acid	0.03 – 0.10	Fernandes et al., 2014
	Cinnamic acid	0.0008 – 0.0009	
Russula virescens (Schaeff.) Fr.	*p*-Hydroxybenzoic acid	22.59	Leal et al., 2013
	Cinnamic acid	15.75	
Sarcodon imbricatus (L.) P. Karst.	*p*-Hydroxybenzoic acid	3.32	Barros et al., 2009
Suillus collinitus (Fr.) Kuntze	Protocatechuic acid	0.52	Vaz et al., 2011b
	p-Hydroxybenzoic acid	1.41	
	Cinnamic acid	0.13	
Suillus granulatus (L.) Roussel	Gallic acid	0.11	Reis et al., 2014a Ribeiro et al., 2006
	p-Hydroxybenzoic acid	0.48	
	Cinnamic acid	0.13	
	Quercetin	0.2 – 1.59	
Suillus luteus (L.: Fries) Gray	Protocatechuic acid	0.47	Reis et al., 2011b Ribeiro et al., 2006
	Cinnamic acid	0.41	
	Quercetin	0.46	
Suillus mediterraneensis (Jacquet. &	Protocatechuic acid	0.14	Vaz et al., 2011b

J.Blum) Redeuilh	*p*-Hydroxybenzoic acid	0.20	
	Cinnamic acid	0.10	
Tricholoma acerbum (Bull.) Quél.	*p*-Hydroxybenzoic acid	2.97	Barros et al., 2009
	Vanillic acid	0.49 – 0.78	
Tricholoma atrosquamosum (Chevall.) Sacc.	*p*-Coumaric acid	7.93	Vaz et al., 2011b
	Cinnamic acid	0.74	
Volvopluteus gloiocephalus (DC.) Vizzini, Contu & Justo	Gallic acid	0.02 – 0.07	Heleno et al., 2015a
	p-Hydroxybenzoic acid	0.003 – 0.09	
	p-Coumaric acid	0.02 – 0.17	
	Cinnamic acid	0.05 – 0.07	
Xerocomus chrysenteron (Bull.) Šutara	Protocatechuic acid	0.54	Heleno et al., 2012a
	p-Hydroxybenzoic acid	0.98	
	p-Coumaric acid	0.55	
	Cinnamic acid	0.55	

Values are presented in mg/100 g of dry weight.

Table S3. Antioxidant properties of Portuguese edible mushroom species.

Mushroom species	**Extract**	**Antioxidant activity mechanism**	**Concentration of reducing substances / EC_{50} values**	**Reference**
Agaricus albertii (Bon.)	Methanolic	Reducing power	18.36 (mg GAE/g extract) 3.04 (EC_{50}; mg/mL)	Reis et al., 2014b
		DPPH radical scavenging activity	10.17 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	3.78 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	0.61 (EC_{50}; mg/mL)	
Agaricus arvensis Schaeff.	Methanolic	Reducing power	2.75 (mg GAE/g extract) 2.86 - 4.20 (EC_{50}; mg/mL)	Barros et al., 2007a, 2008a and 2009
		DPPH radical scavenging activity	3.50 - 48.30 (EC_{50}; mg/mL)	
		Inhibition of erythrocyte hemolysis	> 5 - >50 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	> 5 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	>50 (EC_{50}; mg/mL)	
Agaricus bisporus (J.E. Lange) Imbach	Methanolic	Reducing power	4.49 - 37.33 (mg GAE/g extract) 1.47 - 3.63 (EC_{50}; mg/mL)	Barros et al., 2008a and 2009
		DPPH radical scavenging activity	2.29 - 9.61 (EC_{50}; mg/mL)	Reis et al., 2012b

		Inhibition of erythrocyte hemolysis	> 50 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	4.85 - 21.39 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	1.45 - 46.82 (EC_{50}; mg/mL)	
Agaricus campestres L. ex Fr.	Methanolic	Reducing power	20.94 (mg GAE/g extract)	Pereira et al., 2012
			2.70 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	5.48 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	4.59 (EC_{50}; mg/mL)	
Agaricus comtulus Fr.	Methanolic	Reducing power	24.13 (mg GAE/g extract)	Pereira et al., 2012
			1.29 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	2.22 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	1.08 (EC_{50}; mg/mL)	
Agaricus lutosus (F.H. Møller) F.H. Møller	Methanolic	Reducing power	46.56 (mg GAE/g extract)	Pereira et al., 2012
			0.91 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	2.54 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	0.90 (EC_{50}; mg/mL)	
Agaricus silvaticus Schaeff ex. Secr.	Methanolic	Reducing power	2.08 (EC_{50}; mg/mL)	Barros et al., 2008a
		DPPH radical scavenging activity	5.37 (EC_{50}; mg/mL)	
		Inhibition of erythrocyte hemolysis	22.15 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	3.72 (EC_{50}; mg/mL)	

		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	17.79 (EC_{50}; mg/mL)	
Agaricus silvicola (Vittad.) Peck	Methanolic	Reducing power	6.40 (mg GAE/g extract) 3.24 (EC_{50}; mg/mL)	Barros et al., 2008a and 2009
		DPPH radical scavenging activity	6.39 (EC_{50}; mg/mL)	
		Inhibition of erythrocyte hemolysis	43.75 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	14.75 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	31.97 (EC_{50}; mg/mL)	
Agaricus urinascens var. *excellens* (F.H. Møller) Nauta	Methanolic	Reducing power	15.44 (mg GAE/g extract) 3.71 (EC_{50}; mg/mL)	Reis et al., 2014b
		DPPH radical scavenging activity	13.80 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	4.30 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	6.60 (EC_{50}; mg/mL)	
Amanita caesarea (Scop.) Pers	Methanolic	Reducing power	29.8 (mg GAE/g extract) 1.36 - 1.85 (EC_{50}; mg/mL)	Fernandes et al., 2015 Reis et al., 2011b
		DPPH radical scavenging activity	7.41 - 8 (EC_{50}; mg/mL)	

		β-carotene bleaching inhibition	3.7 - 7.47 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	1.5 (EC_{50}; mg/mL)	
Amanita crocea (Quél. in Bourd.) Singer ex Singer	Methanolic	Reducing power	22.27 (mg GAE/g extract)	Leal et al., 2013
			1.08 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	7.94 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	50.44 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	1.44 (EC_{50}; mg/mL)	
Amanita curtipes E.-J. Gilbert	Methanolic	Reducing power	30.6 (mg GAE/g extract)	Fernandes et al., 2015
			1.53 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	19.0 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	9.8 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	0.7 (EC_{50}; mg/mL)	
Amanita mairei (Foley)	Methanolic	Reducing power	8.94 (mg GAE/ g extract)	Leal et al., 2013
			2.00 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	13.81 (EC_{50}; mg/mL)	

		β-carotene bleaching inhibition	14.10 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	0.66 (EC_{50}; mg/mL)	
Amanita rubescens (Pers. ex Fr.) Gray	Aqueous	DPPH radical scavenging activity	0.304 - 0.990 (IC_{25}; mg/mL)	Ribeiro et al., 2008b
Amanita umbrinolutea (Secr. ex Gillet) Bataille	Methanolic	Reducing power	9.22 (mg GAE/ g extract)	Pereira et al., 2012
			2.71 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	10.02 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	3.69 (EC_{50}; mg/mL)	
Armillaria mellea (Vahl. ex Fr.) Kummer	Ethanolic Polysaccharidic	Reducing power	0.98 – 7.53 (EC_{50}; mg/mL)	Vaz et al., 2011a
		DPPH radical scavenging activity	3.95 - 17.13 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	0.87 - 8.94 (EC_{50}; mg/mL)	
Boletus aereus Bull.	Methanolic	Reducing power	46.05 (mg GAE/ g extract)	Heleno et al., 2011
			0.47 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	0.25 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	0.60 (EC_{50}; mg/mL)	
Boletus armeniacus Quél.	Methanolic	Reducing power	44.66 (mg GAE/ g extract)	Pereira et al., 2012
			0.63 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	1.74 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	0.77 (EC_{50}; mg/mL)	
Boletus edulis Bull.	Aqueous	DPPH radical scavenging activity	0.0774 - 0.184 (IC_{25}; mg/mL)	Ribeiro et al., 2008b

	Methanolic	Reducing power	28.56 – 57 (mg GAE/ g extract)	Fernandes et al., 2013a and 2014 Heleno et al., 2011
		DPPH radical scavenging activity	0.62 - 1.16 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	0.43 – 2.00 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	1.60 - 2.46 (EC_{50}; mg/mL)	
			1.60 – 3.3 (EC_{50}; mg/mL)	
Boletus impolitus Fr.	Methanolic	Reducing power	15.50 (mg GAE/g extract)	Pereira et al., 2012
		DPPH radical scavenging activity	2.04 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	5.81 (EC_{50}; mg/mL)	
			2.04 (EC_{50}; mg/mL)	
Boletus porosporus Imler ex Bon & G. Moreno	Methanolic	Reducing power	20.15 (mg GAE/g extract)	Leal et al., 2013
		DPPH radical scavenging activity	1.58 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	6.97 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	17.08 (EC_{50}; mg/mL)	
			5.49 (EC_{50}; mg/mL)	
Boletus regius (Krombh.)	Methanolic	Reducing power	30.21 (mg GAE/g extract)	Leal et al., 2013
		DPPH radical scavenging activity	0.49 (EC_{50}; mg/mL)	
			2.06 (EC_{50}; mg/mL)	

		β-carotene bleaching inhibition	3.81 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	0.51 (EC_{50}; mg/mL)	
Boletus reticulatus Schaeff.	Methanolic	Reducing power	42.62 (mg GAE/g extract)	Heleno et al., 2011
			0.96 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	0.38 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	1.62 (EC_{50}; mg/mL)	
Bovista aestivalis (Bonord.) Demoulin	Methanolic	Reducing power	50.91 (mg GAE/g extract)	Pereira et al., 2012
			0.51 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	2.05 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	0.61 (EC_{50}; mg/mL)	
Bovista nigrescens (Pers.)	Methanolic	Reducing power	26.50 (mg GAE/g extract)	Pereira et al., 2012
			1.21 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	4.62 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	1.91 (EC_{50}; mg/mL)	
Calocybe gambosa (Fr.) Donk	Ethanolic Polysaccharidic	Reducing power	2.38 – 11.46 (EC_{50}; mg/mL)	Vaz et al., 2011a
		DPPH radical scavenging activity	7.08 – 34.60 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	7.57 – 8.17 (EC_{50}; mg/mL)	
Calvatia utriformis (Bull. ex Pers.) Jaap	Methanolic	Reducing power	1.16 (EC_{50}; mg/mL)	Grangeia et al., 2011
		DPPH radical scavenging activity	0.68 (EC_{50}; mg/mL)	

		β-carotene bleaching inhibition	8.40 (EC_{50}; mg/mL)	
Cantharellus cibarius Fr.	Methanolic	Reducing power	1.75 (mg GAE/g extract)	Barros et al., 2008b and 2009
			8.72 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	19.65 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	8.40 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	8.59 (EC_{50}; mg/mL)	
Chlorophyllum rhacodes (Vittadini) Vellinga	Methanolic	Reducing power	22.77 (mg GAE/g extract)	Pereira et al., 2012
			2.22 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	5.32 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	2.33 (EC_{50}; mg/mL)	
Clavariadelphus pistillaris (L.) Donk	Methanolic	Reducing power	48.10 (mg GAE/g extract)	Pereira et al., 2012
			0.70 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	1.30 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	1.94 (EC_{50}; mg/mL)	
Clavariadelphus truncates (Quél.) Donk	Methanolic	Reducing power	7.66 (mg GAE/g extract)	Pereira et al., 2012
			1.33 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	2.74 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	2.35 (EC_{50}; mg/mL)	
Clitocybe alexandri (Gillet)	Ethanolic	Reducing power	1.5 – 6.3 (mg GAE/g extract)	Heleno et al., 2010

Gillet	Methanolic Polysaccharidic	DPPH radical scavenging activity β-carotene bleaching inhibition	0.9 - 7.0 (EC_{50}; mg/mL) 2.5 - 28.7 (EC_{50}; mg/mL) 1.2 - 4.5 (EC_{50}; mg/mL)	Vaz et al., 2010
Clitocybe costata Kummer & Romagnesi	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	13.71 (mg GAE/g extract) 1.66 (EC_{50}; mg/mL) 10.56 (EC_{50}; mg/mL) 3.22 (EC_{50}; mg/mL)	Pereira et al., 2012
Clitocybe gibba (Pers.) Kumm	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	25.26 (mg GAE/g extract) 1.46 (EC_{50}; mg/mL) 10.61 (EC_{50}; mg/mL) 4.00 (EC_{50}; mg/mL)	Pereira et al., 2012
Clitocybe odora (Fr.) P. Kumm.	Ethanolic Polysaccharidic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	0.94 – 3.63 (EC_{50}; mg/mL) 3.56 – 6.77 (EC_{50}; mg/mL) 0.27 – 1.36 (EC_{50}; mg/mL)	Vaz et al., 2011a
Clitopilus prunulus (Scop. ex Fr.) P. Kumm.	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	3.36 (EC_{50}; mg/mL) 1.75 (EC_{50}; mg/mL) 12.06 (EC_{50}; mg/mL)	Grangeia et al., 2011
Coprinopsis atramentaria (Bull.) Redhead, Vilgalys & Moncalvo	Phenolic Polysaccharidic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	33.58 (mg GAE/g extract) 0.88 – 1.29 (EC_{50}; mg/mL) 2.48 – 3.87 (EC_{50}; mg/mL) 0.81 – 1.03 (EC_{50}; mg/mL)	Heleno et al., 2012a

		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	1.01 – 1.09 (EC_{50}; mg/mL)	
	Methanol/water (80:20, *v/v*)	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	1.11 (EC_{50}; mg/mL) 4.62 (EC_{50}; mg/mL) 5.28 (EC_{50}; mg/mL)	Ribeiro et al., 2015
Coprinus comatus (O.F.Müll.) Pers.	Ethanolic Polysaccharidic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	1.47 – 4.67 (EC_{50}; mg/mL) 2.56 – 7.31 (EC_{50}; mg/mL) 1.26 – 7.43 (EC_{50}; mg/mL)	Vaz et al., 2011a
Cortinarius glaucopus (Schaeff.) Fr.	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	2.80 (mg GAE/g extract) 16.59 (EC_{50}; mg/mL) 1.73 (EC_{50}; mg/mL)	Heleno et al., 2010
Cortinarius praestans (Cordier) Gillet	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	17.81 (mg GAE/g extract) 1.70 (EC_{50}; mg/mL) 3.04 (EC_{50}; mg/mL) 2.04 (EC_{50}; mg/mL)	Pereira et al., 2012
Cortinarius violaceus (L.: Fr.) Gray	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	2.36 (EC_{50}; mg/mL) 15.70 (EC_{50}; mg/mL) 5.73 (EC_{50}; mg/mL)	Reis et al., 2011b
Flammulina velutipes (Curtis) Singer	Methanolic	Reducing power	12.98 (mg GAE/g extract) 1.94 (EC_{50}; mg/mL)	Pereira et al., 2012

		DPPH radical scavenging activity	6.19 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	1.12 (EC_{50}; mg/mL)	
Fistulina hepatica (Schaeff.) With.	Methanolic	Reducing power	4.44 (mg GAE/g extract)	Heleno et al., 2010
		DPPH radical scavenging activity	5.32 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	0.94 (EC_{50}; mg/mL)	
Ganoderma lucidum (Curtis) P. Karst.	Phenolic Polysaccharidic	Reducing power	28.64 – 55.53 (mg GAE/g extract) 0.62 – 0.81 (EC_{50}; mg/mL)	Heleno et al., 2012b
		DPPH radical scavenging activity	0.14 – 0.22 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	0.26 – 9.03 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	0.10 – 1.21 (EC_{50}; mg/mL)	
Gyromitra esculenta (Pers. ex Pers.) Fr.	Methanolic	Reducing power	27.16 (mg GAE/g extract) 6.82 (EC_{50}; mg/mL)	Leal et al., 2013
		DPPH radical scavenging activity	12.66 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	9.15 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	3.95 (EC_{50}; mg/mL)	
Helvella lacunose (Afzel.)	Methanolic	Reducing power	13.66 (mg GAE/g extract)	Leal et al., 2013

		DPPH radical scavenging activity	17.60 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	26.92 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	6.53 (EC_{50}; mg/mL)	
			1.13 (EC_{50}; mg/mL)	
Hericium erinaceus (Bull.) Persoon	Methanolic	Reducing power	13.41 (mg GAE/g extract)	Heleno et al., 2015b
		DPPH radical scavenging activity	3.47 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	24.53 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	0.27 (EC_{50}; mg/mL)	
			1.37 (EC_{50}; mg/mL)	
Hericium coralloides (Scop.) Pers.	Methanolic	Reducing power	16.29 (mg GAE/g extract)	Heleno et al., 2015b
		DPPH radical scavenging activity	1.63 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	22.53 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	0.28 (EC_{50}; mg/mL)	
			0.74 (EC_{50}; mg/mL)	
Hydnum repandum L.	Methanolic	Reducing power	0.51 – 6.80 (mg GAE/g extract)	Heleno et al., 2010

		DPPH radical scavenging activity β-carotene bleaching inhibition Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	2.47 (EC_{50}; mg/mL) 30.00 – 31.00 (EC_{50}; mg/mL) 3.80 - 28.72 (EC_{50}; mg/mL) 0.9 (EC_{50}; mg/mL)	Fernandes et al., 2013a
Hygrophoropsis aurantiaca (Wulfen) Maire	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	7.90 (mg GAE/g extract) 1.20 (EC_{50}; mg/mL) 0.71 (EC_{50}; mg/mL)	Heleno et al., 2010
Hygrophorus chrysodon (Fr.) Fr.	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	4.58 (mg GAE/g extract) 7.82 (EC_{50}; mg/mL) 20.02 (EC_{50}; mg/mL) 5.95 (EC_{50}; mg/mL)	Pereira et al., 2012
Hygrophorus pustulatus (Pers. : Fr.) Fries	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	2.61 (EC_{50}; mg/mL) 0.74 (EC_{50}; mg/mL) 1.28 (EC_{50}; mg/mL)	Grangeia et al., 2011
Hypholoma capnoides (Fr.) P. Kumm.	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	1.71 (mg GAE/g extract) 20.85 (EC_{50}; mg/mL) 2.90 (EC_{50}; mg/mL)	Heleno et al., 2010
Laccaria amethystina (Huds.) Cooke	Methanolic	Reducing power DPPH radical scavenging activity	2.85 (mg GAE/g extract) 15.72 (EC_{50}; mg/mL)	Heleno et al., 2010

		β-carotene bleaching inhibition	1.23 (EC_{50}; mg/mL)	
Laccaria laccata (Scop.) Cooke	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	1.59 (mg GAE/g extract) 21.95 (EC_{50}; mg/mL) 3.69 (EC_{50}; mg/mL)	Heleno et al., 2010
Lactarius aurantiacus (Pers.) Gray	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	0.58 (mg GAE/g extract) 30.00 (EC_{50}; mg/mL) 7.48 (EC_{50}; mg/mL)	Heleno et al., 2010
Lactarius deliciosus (L. ex Fr.) S.F.Gray	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	2.95 – 10.66 (mg GAE/g extract) 3.42 – 6.69 (EC_{50}; mg/mL) 8.52 – 20.54 (EC_{50}; mg/mL) 3.74 – 3.76 (EC_{50}; mg/mL) 26.40 – 29.63 (EC_{50}; mg/mL)	Barros et al., 2007c and 2009 Ferreira et al., 2007
Lactarius piperatus (L.) Pers.	Methanolic	Reducing power DPPH radical scavenging activity Inhibition of erythrocyte hemolysis β-carotene bleaching inhibition	2.03 – 5.76 (mg GAE/g extract) 2.29 – 5.50 (EC_{50}; mg/mL) 5.19 - 23.44 (EC_{50}; mg/mL) 12.14 - > 50 (EC_{50}; mg/mL) 12.08 – 45.06 (EC_{50}; mg/mL)	Barros et al., 2007d and 2009
Lactarius salmonicolor R.Heim & Leclair	Methanolic	Reducing power DPPH radical scavenging activity	4.14 (mg GAE/g extract) 7.80 (EC_{50}; mg/mL)	Heleno et al., 2010

		β-carotene bleaching inhibition	1.01 (EC_{50}; mg/mL)	
Lactarius volemus (Fr.) Fr.	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	2.42 (EC_{50}; mg/mL) 21.68 (EC_{50}; mg/mL) 9.08 (EC_{50}; mg/mL)	Reis et al., 2011b
Lentinula edodes (Berk.) Pegler	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	8.84 (mg GAE/g extract) 2.62 (EC_{50}; mg/mL) 6.43 (EC_{50}; mg/mL) 3.92 (EC_{50}; mg/mL) 1.64 (EC_{50}; mg/mL)	Reis et al., 2012b
Lepista inversa (Scop. Fr.) Pat.	Ethanolic Methanolic Polysaccharidic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	3.6 – 10.8 (mg GAE/g extract) 0.7 - 2.9 (EC_{50}; mg/mL) 1.8 - 10.6 (EC_{50}; mg/mL) 0.9 - 1.1 (EC_{50}; mg/mL)	Heleno et al., 2010 Vaz et al., 2010
Lepista nuda (Bull.) H.E. Bigelow & A.H. Sm.	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition Inhibition of lipid peroxidation using thiobarbituric acid reactive substances	6.31 – 20.54 (mg GAE/g extract) 1.50 – 3.53 (EC_{50}; mg/mL) 4.41 – 8.73 (EC_{50}; mg/mL) 4.21 – 9.48 (EC_{50}; mg/mL) 1.75 – 5.80 (EC_{50}; mg/mL)	Barros et al., 2008c and 2009 Pinto et al., 2013

		(TBARS)		
Lepista sordida (Fr.) Sing.	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	4.10 (mg GAE/g extract) 9.82 (EC_{50}; mg/mL) 1.03 (EC_{50}; mg/mL)	Heleno et al., 2010
Leucoagaricus leucothites (Vittad.) Wasser	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	15.75 (mg GAE/g extract) 3.28 (EC_{50}; mg/mL) 11.33 (EC_{50}; mg/mL) 1.00 (EC_{50}; mg/mL)	Pereira et al., 2012
Leucopaxillus giganteus (Sowerby) Singer	Methanolic	Reducing power DPPH radical scavenging activity Inhibition of erythrocyte hemolysis β-carotene bleaching inhibition	6.29 (mg GAE/g extract) 1.71 (EC_{50}; mg/mL) 1.44 (EC_{50}; mg/mL) 1.80 (EC_{50}; mg/mL) 2.00 (EC_{50}; mg/mL)	Barros et al., 2007a and 2009
Lycoperdon echinatum Pers.	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	1.61 (EC_{50}; mg/mL) 1.04 (EC_{50}; mg/mL) 1.24 (EC_{50}; mg/mL)	Grangeia et al., 2011
Lycoperdon molle Pers.	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition Inhibition of lipid peroxidation using	11.48 (mg GAE/g extract) 2.27 (EC_{50}; mg/mL) 3.23 (EC_{50}; mg/mL) 1.92 (EC_{50}; mg/mL) 3.31 (EC_{50}; mg/mL)	Barros et al., 2008c and 2009

		thiobarbituric acid reactive substances (TBARS)		
Lycoperdon perlatum Pers.	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	10.57 (mg GAE/g extract) 2.96 (EC_{50}; mg/mL) 3.95 (EC_{50}; mg/mL) 2.49 (EC_{50}; mg/mL) 4.64 (EC_{50}; mg/mL)	Barros et al., 2008c and 2009
Lycoperdon umbrinum Pers.	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	27.02 (mg GAE/g extract) 1.27 (EC_{50}; mg/mL) 3.45 (EC_{50}; mg/mL) 2.24 (EC_{50}; mg/mL)	Pereira et al., 2012
Lyophyllum decastes (Fr. ex Fr.) Sing.	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	0.97 (EC_{50}; mg/mL) 0.29 (EC_{50}; mg/mL) 0.98 (EC_{50}; mg/mL)	Grangeia et al., 2011
Macrolepiota excoriate (Schaeff.) Wasser	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	1.84 (EC_{50}; mg/mL) 1.32 (EC_{50}; mg/mL) 1.23 (EC_{50}; mg/mL)	Grangeia et al., 2011
Macrolepiota mastoidea (Fr.) Singer	Methanolic	Reducing power	2.69 – 3.08 (mg GAE/g extract) 4.35 – 4.44 (EC_{50}; mg/mL)	Barros et al., 2007c

		DPPH radical scavenging activity	8.18 – 8.49 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	6.48 – 8.92 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	24.20 – 34.42 (EC_{50}; mg/mL)	
Macrolepiota procera (Scop.) Singer	Methanolic	Reducing power	3.17 (mg GAE/g extract)	Barros et al., 2007c and 2009 Fernandes et al., 2013b
		DPPH radical scavenging activity	4.18 – 4.49 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	5.38 – 6.95 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	5.19 – 6.23 (EC_{50}; mg/mL)	
			> 50 (EC_{50}; mg/mL)	
Morchella esculenta Fr.	Methanolic	Reducing power	34.64 (mg GAE/g extract)	Heleno et al., 2013a
		DPPH radical scavenging activity	6.34 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	6.06 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	0.81 (EC_{50}; mg/mL)	
			1.01 (EC_{50}; mg/mL)	
Pleurotus eryngii (D.C.:Fr.) Quél.	Methanolic	Reducing power	7.14 - 18.43 (mg GAE/g extract)	Reis et al., 2012b and 2014b
			2.17 - 3.72 (EC_{50}; mg/mL)	

		DPPH radical scavenging activity	8.67 - 9.21 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	4.68 - 14.90 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	1.59 - 3.95 (EC_{50}; mg/mL)	
Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm.	Methanolic	Reducing power	12.54 (mg GAE/g extract) 3.31 (EC_{50}; mg/mL)	Reis et al., 2012b
		DPPH radical scavenging activity	6.54 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	2.74 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	2.58 (EC_{50}; mg/mL)	
Ramaria aurea (Schaeff.) Quél.	Methanolic	Reducing power	8.46 (mg GAE/g extract) 0.99 (EC_{50}; mg/mL)	Pereira et al., 2012
		DPPH radical scavenging activity	3.70 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	2.46 (EC_{50}; mg/mL)	
Russula aurea Pers.	Methanolic	Reducing power	12.23 (mg GAE/g extract) 2.91 (EC_{50}; mg/mL)	Leal et al., 2013
		DPPH radical scavenging activity	11.34 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	9.70 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using	2.98 (EC_{50}; mg/mL)	

		thiobarbituric acid reactive substances (TBARS)		
	Aqueous	DPPH radical scavenging activity	0.760 - 0.936 (IC_{25}; mg/mL)	Ribeiro et al., 2008b
Russula cyanoxantha (Schaeff.) Fr.	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	2.26 (EC_{50}; mg/mL) 0.69 (EC_{50}; mg/mL) 0.98 (EC_{50}; mg/mL)	Grangeia et al., 2011
Russula delica Fr.	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	2.28 - 47 (mg GAE/g extract) 0.26 (EC_{50}; mg/mL) 4.3 - 20.53 (EC_{50}; mg/mL) 0.53 - 2.28 (EC_{50}; mg/mL) 1.23 (EC_{50}; mg/mL)	Heleno et al., 2010 Fernandes et al., 2014
Russula olivacea (Schaeff.) Fr.	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	7.85 (EC_{50}; mg/mL) 1.47 (EC_{50}; mg/mL) 1.95 (EC_{50}; mg/mL)	Grangeia et al., 2011
Russula vesca Fr.	Methanolic	Reducing power DPPH radical scavenging activity β-carotene bleaching inhibition	6.61 (mg GAE/g extract) 3.91 (EC_{50}; mg/mL) 0.91 (EC_{50}; mg/mL)	Heleno et al., 2010
Russula virescens (Schaeff.) Fr.	Methanolic	Reducing power	14.05 (mg GAE/g extract) 8.24 (EC_{50}; mg/mL)	Leal et al., 2013

		DPPH radical scavenging activity	30.21 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	4.28 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	0.23 (EC_{50}; mg/mL)	
Sarcodon imbricatus (L.) P. Karst.	Methanolic	Reducing power	2.22 – 3.06 (mg GAE/g extract) 2.79 – 5.94 (EC_{50}; mg/mL)	Barros et al., 2007a, 2007c and 2009
		DPPH radical scavenging activity	1.67 – 10.98 (EC_{50}; mg/mL)	
		Inhibition of erythrocyte hemolysis	> 5 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	3.53 – 4.45 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	38.17 – 41.14 (EC_{50}; mg/mL)	
Suillus collinitus (Fr.) Kuntze	Methanolic	Reducing power	3.16 (mg GAE/g extract)	Heleno et al., 2010
		DPPH radical scavenging activity	14.05 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	1.20 (EC_{50}; mg/mL)	
Suillus granulatus (L.) Roussel	Aqueous	DPPH radical scavenging activity	0.184 - 196 (IC_{25}; mg/mL)	Ribeiro et al., 2008b
	Methanolic	Reducing power	40.78 (mg GAE/g extract) 0.57 (EC_{50}; mg/mL)	Reis et al., 2014a
		DPPH radical scavenging activity	0.98 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	0.45 (EC_{50}; mg/mL)	

		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	0.03 (EC_{50}; mg/mL)	
Suillus luteus (L.: Fries) Gray	Methanolic	Reducing power	0.75 (EC_{50}; mg/mL)	Reis et al., 2011b
		DPPH radical scavenging activity	1.92 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	0.61 (EC_{50}; mg/mL)	
	Methanol/water (80:20, *v/v*)	Reducing power	0.97 (EC_{50}; mg/mL)	Ribeiro et al., 2015
		DPPH radical scavenging activity	2.86 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	1.64 (EC_{50}; mg/mL)	
Suillus mediterraneensis (Jacquet. & J.Blum) Redeuilh	Methanolic	Reducing power	0.81 (mg GAE/g extract)	Heleno et al., 2010
		DPPH radical scavenging activity	2.90 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	0.81 (EC_{50}; mg/mL)	
Suillus variegatus (Sw.) Kuntze	Methanolic	Reducing power	58.14 (mg GAE/g extract)	Pereira et al., 2012
			0.52 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	0.86 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	1.00 (EC_{50}; mg/mL)	
Tricholoma acerbum (Bull.) Quél.	Methanolic	Reducing power	5.53 (mg GAE/g extract)	Barros et al., 2008c and 2009
			3.27 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	3.60 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	5.89 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using	6.20 (EC_{50}; mg/mL)	

		thiobarbituric acid reactive substances (TBARS)		
Tricholoma portentosum (Fr.) Quél.	Methanolic	Reducing power	3.91 – 10.80 (mg GAE/g extract)	Ferreira et al., 2007
			3.12 – 4.82 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	22.9 – >50 (EC_{50}; mg/mL)	
Xerocomus chrysenteron (Bull.) Šutara	Phenolic Polysaccharidic	Reducing power	36.28 (mg GAE/g extract)	Heleno et al., 2012b
			1.28 – 2.90 (EC_{50}; mg/mL)	
		DPPH radical scavenging activity	2.06 – 11.31 (EC_{50}; mg/mL)	
		β-carotene bleaching inhibition	0.95 – 4.43 (EC_{50}; mg/mL)	
		Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)	0.44 – 4.94 (EC_{50}; mg/mL)	

GAE - Gallic acid equivalents; EC_{50}: extract concentration corresponding to 50% antioxidant activity or 0.5 absorbance for the reducing power assay.

Table S4. Antibacterial properties of Portuguese edible mushroom species.

Mushroom species	**Extract**	**Pathogens against which the antimicrobial activity is exerted**	**MIC (mg/mL)** **MBC (mg/mL)**	**Reference**
Agaricus bisporus (J.E. Lange) Imbach	Methanolic	*Bacillus subtilis*	MIC: 0.005	Barros et al., 2008b
Agaricus silvicola (Vittad.) Peck	Methanolic	*Bacillus cereus*	MIC: 0.005	Barros et al., 2008b
		Bacillus subtilis	MIC: 0.050	
		Staphylococus aureus	MIC: 0.005	
Boletus edulis Bull.	Methanolic	*Staphylococus aureus*	MIC: 0.005	Barros et al., 2008b
Cantharellus cibarius Fr.	Methanolic	*Bacillus cereus*	MIC: 0.005	Barros et al., 2008b and 2008c
		Bacillus subtilis	MIC: 0.005	
		Staphylococus aureus	MIC: 0.005 - 0.050	
Ganoderma lucidum (Curtis) P. Karst.	Methanolic	*Bacillus cereus*	MIC: 0.013 MBC: 0.035	Heleno et al., 2013b
		Enterobacter cloacae	MIC: 0.350 MBC: 0.750	
		Escherichia coli	MIC: 0.350 MBC: 0.750	
		Listeria monocytogenes	MIC: 0.300 MBC: 0.750	
		Micrococcus flavus	MIC: 0.500 MBC: 0.750	
		Pseudomonas aeruginosa	MIC: 0.750 MBC: 1.500	
		Salmonella typhimurium	MIC: 0.350 MBC: 0.750	
		Staphylococus aureus	MIC: 0.025	

			MBC: 0.035	
Lactarius deliciosus (L. ex Fr.) S.F.Gray	Methanolic	*Bacillus cereus*	MIC: 0.5 – 50	Barros et al., 2007e
		Bacillus subtilis	MIC: 0.5 – 300	
		Candida albicans	MIC: 50 – 100	
		Cryptococcus neoformans	MIC: 10 – 50	
		Pseudomonas aeruginosa	MIC: 5 – 300	
		Staphylococus aureus	MIC: 5 – 50	
Lactarius piperatus (L.) Pers.	Methanolic	*Bacillus cereus*	MIC: 0.5	Barros et al., 2007b
		Bacillus subtilis	MIC: 0.5	
		Escherichia coli	MIC: 5	
		Klebsiella pneumoniae	MIC: 0.5 – 5	
		Pseudomonas aeruginosa	MIC: 0.5 – 5	
		Staphylococus aureus	MIC: 0.5	
Lepista nuda (Bull.) H.E. Bigelow & A.H. Sm.	Methanolic	*Bacillus cereus*	MIC: 0.005	Barros et al., 2008c
		Bacillus subtilis	MIC: 0.005	
		Staphylococus aureus	MIC: 0.005	
Phellinus linteus (Berkeley & Curtis) Teng	Methanolic	*Bacillus cereus*	MIC: 0.032 MBC: 0.043	Reis et al., 2014c
		Enterobacter cloacae	MIC: 0.048 MBC: 0.104	
		Escherichia coli	MIC: 0.072 MBC: 0.12	
		Listeria monocytogenes	MIC: 0.048 MBC: 0.18	
		Micrococcus flavus	MIC: 0.048 MBC: 0.13	
		Pseudomonas aeruginosa	MIC: 0.13	

			MBC: 0.17	
		Salmonella typhimurium	MIC: 0.095 MBC: 0.19	
		Staphylococus aureus	MIC: 0.12 MBC: 019	
	Ethanolic	*Bacillus cereus*	MIC: 0.077 MBC: 0.10	
		Enterobacter cloacae	MIC: 0.12 MBC: 0.22	
		Escherichia coli	MIC: 0.17 MBC: 0.23	
		Listeria monocytogenes	MIC: 0.022 MBC: 0.40	
		Micrococcus flavus	MIC: 0.15 MBC: 0.18	
		Pseudomonas aeruginosa	MIC: 0.047 MBC: 0.12	
		Salmonella typhimurium	MIC: 0.12 MBC: 0.37	
		Staphylococus aureus	MIC: 0.10 MBC: 0.22	
Sarcodon imbricatus (L.) P. Karst.	Methanolic	*Bacillus cereus* *Cryptococcus neoformans*	MIC: 10 – 300 MIC: 300	Barros et al., 2007e
Suillus granulatus (L.) Roussel	Methanolic	*Bacillus cereus*	MIC: 0.10 MBC: 0.20	Reis et al., 2014a
		Enterobacter cloacae	MIC: 0.15 MBC: 0.2	
		Escherichia coli	MIC: 0.15	

			MBC: 0.2	
		Listeria monocytogenes	MIC: 0.2 MBC: 0.4	
		Micrococcus flavus	MIC: 0.2 MBC: 0.4	
		Pseudomonas aeruginosa	MIC: 0.15 MBC: 0.2	
		Salmonella typhimurium	MIC: 0.15 MBC: 0.2	
		Staphylococus aureus	MIC: 0.15 MBC: 0.2	
Tricholoma acerbum (Bull.) Quél.	Methanolic	*Bacillus subtilis*	MIC: 0.5	Barros et al., 2008b
Tricholoma portentosum (Fr.) Quél.	Methanolic	*Bacillus cereus*	MIC: 100 – 300	Barros et al., 2007e
		Bacillus subtilis	MIC: 300	
		Cryptococcus neoformans	MIC: 300	

MIC – minimum inhibitory concentration; MBC – minimum bactericidal concentration.

Table S5. Antifungal properties of Portuguese edible mushroom species.

Mushroom species	Extract	Pathogens against which the antifungal activity is exerted	MIC (mg/mL) MFC (mg/mL)	Reference
Ganoderma lucidum (Curtis) P. Karst.	Methanolic	*Aspergillus fumigatus*	MIC: 1.5 MFC: 3.0	Heleno et al., 2013b
		Aspergillus niger	MIC: 1.5 MFC: 3.0	
		Aspergillus ochraceus	MIC: 0.75 MFC: 1.5	
		Aspergillus versicolor	MIC: 0.1 MFC: 4.5	
		Penicillium funiculosum	MIC: 0.09 MFC: 1.5	
		Penicillium ochrochloron	MIC: 0.35 MFC: 0.7	
		Penicillium verrucosum	MIC: 1.5 MFC: 3.0	
		Trichoderma viride	MIC: 0.005 MFC: 0.1	
Suillus granulatus (L.) Roussel	Methanolic	*Aspergillus fumigatus*	MIC: 0.45 MFC: 0.8	Reis et al., 2014a
		Aspergillus niger	MIC: 0.05 MFC: 0.1	
		Aspergillus ochraceus	MIC: 0.1 MFC: 0.2	
		Aspergillus versicolor	MIC: 0.1	

		MFC: 0.2
	Penicillium funiculosum	MIC: 0.05 MFC: 0.1
	Penicillium ochrochloron	MIC: 0.075 MFC: 0.1
	Penicillium verrucosum var. *cyclopium*	MIC: 0.1 MFC: 0.4
	Trichoderma viride	MIC: 0.075 MFC: 0.1

MIC – minimum inhibitory concentration; MFC – minimum fungicidal concentration.

ANNEX 2. PUBLISHED WORK FROM THIS RESEARCH

Book Chapter

[1] Filipa S. Reis, Patricia Morales, Isabel C.F.R. Ferreira, M. Helena Vasconcelos. (2016). Mushrooms as a source of compounds that induce programmed cell death in tumour cells, in J.N. Govil & M. Pathak (Eds.), Phytotherapeutics II, Vol.43 of the Series Recent Progress in Medicinal Plants, Studium Press LLC: U.S.A., 85-119.

Published Articles

[1] Filipa S. Reis, Sandrina A. Heleno, Lillian Barros, Maria João Sousa, Anabela Martins, Celestino Santos-Buelgas, Isabel C.F.R. Ferreira. (2011). Toward the antioxidant and chemical characterization of mycorrhizal mushrooms from Northeast Portugal. *Journal of Food Science*, 76, 824-830. (DOI: 10.1111/j.1750-3841.2011.02251.x)

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ANNEX 3. FIGURE CAPTIONS

ANNEX 4. TABLE CAPTIONS

ANNEX 5. EQUATION CAPTIONS