

Wild mushrooms and their mycelia as sources of bioactive compounds: antioxidant, anti-inflammatory and cytotoxic properties

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ABBREVIATIONS LIST

5-LOX: 5-Lipoxygenase

ANOVA: One way analysis of variance

CAT: Catalase

COMT: Caffeic/5-hydroxyferulic acid-*O*-transferase

COX: Cyclooxygenase

COX-2: Cyclooxygenase-2

DAD: Diode array detector

DMEM: Dulbecco's modified Eagle's minimum essential medium

DNA: Deoxyribonucleic acid

DPPH: 2,2-Diphenyl-1-picrylhydrazyl radical

Dw: Dry weight

EC₅₀: Extract concentration corresponding to 50% of antioxidant activity

ECACC: European collection of animal cell culture

ETS: Electron transport system

ECM: Ectomycorrhizal

FBS: Fetal bovine serum

Fw: Fresh weight

GAE: Gallic acid equivalents

GI₅₀: Extract concentration that inhibited 50% of the net cell growth

GPx: Glutathione peroxidase

Gred: Glutathione reductase

GSH: Glutathione

GSNO: S-nitrosoglutathione

GS-SG: Glutathione disulphide

GST: Glutathione-S-transferases

HBSS: Hank's balanced salt solution

HCT15: Colon carcinoma cell line

HeLa: Henrietta Lacks human cervical carcinoma cell line

HepG2: Hepatocellular carcinoma cell line

HMW: High molecular weight

HPLC: High performance liquid chromatography

HPLC-DAD: High performance liquid chromatography with a diode array detector

HPLC-UV: High performance liquid chromatography coupled to an ultraviolet detector

HSD: Tukey's honestly significant difference

HO•: Hydroxyl radical

HO⁻: Hydroxyl ion

H₂O₂: Hydrogen peroxide

ICAM-1: Intercellular adhesion molecule-1

IL-1 β: Interleukin 1β

IL-6: Interleukin 6

IL-8: Interleukin 8

iMMN: Incomplete melin-norkans medium

iNOS: Inducible nitric oxide synthase

L•: Lipidic radical

LH: Membrane lipids

LMW: Low molecular weight

LO•: Lipid alkoxyl radical

LOH: Alcohols

LOO•: Peroxyl radical

LOOH: Hydroperoxide lipid

LPS: Lipopolysaccharide

MCF-7: Breast adenocarcinoma cell line

MDA: Malondialdehyde

MMN: Melin–norkans medium

NADPH: Nicotinamide adenine dinucleotide reduced

NADP⁺: Nicotidamide adenine dinucleotide phosphate: oxidized

NCI-H460: Human lung carcinoma cell line

NF-KB: Nuclear factor kappa B

NED: N-(1-naphthyl) ethylenediamine hydrochloride

NOS: Nitric oxide synthase

NO: Nitric oxide

NO•: Nitric oxide radical

NSAIDs: Nonsteroidal anti-inflammatory drugs

PAL: Phenylalanine ammonialyase

PDA: Potato dextrose agar medium

PDB: Potato dextrose broth

PLP2: Non- tumor primary culture of porcine liver cells

PGE2: Prostaglandin E2

R: Non-radicals

R[•]: Radicals

RNS: Nitrogen reactive species

ROS: Oxygen reactive species

RSA: Radical scavenging activity

SRB: Sulphorodamine B

SOD: Superoxide dismutase

TAL: Tyrosine ammonia lyase

TBARS: Thiobarbituric acid reactive substances

TBA: Thiobarbituric acid

TCA: Trichloroacetic acid

TNF- α : Tumour necrosis factor α

O₂^{•-}: Superoxide anion

Vit. C: Vitamin C

Vit. C[•]: Vitamin C radical

Vit. E: Vitamin E

Vit. E[•]: Vitamin E radical

UV: Ultraviolet

UV-Vis: Ultraviolet visible detector

UFLC: Ultra-fast liquid chromatography

w/v: Weight/Volume

ABSTRACT

Mushrooms are an important source of natural compounds with acknowledged bioactivity. *Pleurotus eryngii* (DC.) Quél., in particular, is widely recognized for its organoleptic quality and favorable health effects, being commercially produced in great extent. On the other hand, *Suillus bellinii* (Inzenga) Watling is an ectomycorrhizal symbiont, whose main properties were only reported in a scarce number of publications. Some current trends point toward using the mycelia and the culture media as potential sources of bioactive compounds, in addition to the fruiting bodies. Accordingly, *P. eryngii* and *S. bellinii* were studied for their composition in phenolic acids and sterols, antioxidant capacity (scavenging DPPH radicals, reducing power, β -carotene bleaching inhibition and TBARS formation inhibition), anti-inflammatory effect (by down-regulating LPS-stimulated NO in RAW264.7 cells) and anti-proliferative activity (using MCF-7, NCI-H460, HeLa, HepG2 and PLP2 cell lines). Overall, *S. bellinii* mycelia showed higher contents of ergosterol and phenolic compounds (which were also detected in higher quantity in its fruiting body) and stronger antioxidant activity than *P. eryngii*. On the other hand, *P. eryngii* mycelia showed anti-inflammatory (absent in *S. bellinii* mycelia) and a cytotoxicity similar (sometimes superior) to its fruiting bodies, in opposition to *S. bellinii*, whose mycelia presented a decreased anti-proliferative activity. Furthermore, the assayed species showed differences in the growth rate and yielded biomass of their mycelia, which should also be considered in further applications.

Keywords: *Pleurotus eryngii* (DC.) Quél., *Suillus bellinii* (Inzenga) Watling, ergosterol, phenolic acids, antioxidant activity, anti-inflammatory activity, anti-proliferative activity.

RESUMO

Os cogumelos são uma importante fonte de compostos naturais com reconhecida bioatividade. *Pleurotus eryngii* (DC.) Quél., em particular, é largamente reconhecido pela sua qualidade organolética e benefícios para a saúde, sendo comercialmente produzido em larga escala.

Por outro lado, *Suillus bellinii* (Inzenga) Watling é um fungo simbiote ectomicorrízico, cujas propriedades estão pouco documentadas. A produção de micélio através da cultura *in vitro* revela-se como uma fonte potencial de obtenção de compostos bioativos, para além da produção pelos carpóforos. Assim sendo, *P. eryngii* e *S. bellinii* foram estudados na sua composição em ácidos fenólicos e esteróis, capacidade antioxidante (capacidade de captação de radicais de DPPH; poder redutor, inibição do branqueamento do β -caroteno e Inibição da formação de TBARS), capacidade anti-inflamatória (através da regulação negativa da NO estimulada pela LPS na linha celular RAW264.7) e a atividade anti-proliferativa (nas linhas celulares MCF-7, NCI-H460, HeLa, HepG2 e PLP2). O micélio de *S. bellinii* mostrou maiores conteúdos de ergosterol e de compostos fenólicos (que também foram detetados em maiores quantidades).

Por outro lado, o micélio de *P. eryngii* apresentou capacidade anti-inflamatória e citotoxicidade similares (e por vezes superiores) ao corpo frutífero, ao contrário do que acontece com *S. bellinii*, cujo micélio apresenta uma atividade anti-proliferativa diminuída.

Além disso, as espécies estudadas apresentam diferenças nas taxas de crescimento e de produção de biomassa dos seus micélios, que tem que ser considerada em futuras aplicações.

Palavras-chave: *Pleurotus eryngii* (DC.) Quél., *Suillus bellinii* (Inzenga) Watling, ergosterol, ácidos fenólicos, atividade antioxidante, atividade anti-inflamatória, atividade anti-proliferativa.

CHAPTER 1. FRAMEWORK

The number of mushroom species on earth is evaluated to be 140.000, proposing that only 10% are already known (Ferreira et al., 2010).

For several centuries, wild mushrooms have been part of the normal human diet, and also extensively consumed due to their organoleptic properties including distinctive flavor, texture, and taste. Edible wild mushrooms have been described as source of chemical and nutritional properties. They are rich in minerals, water (80 to 90%), protein, fiber and vitamins (thiamine, riboflavin, ascorbic acid, vitamin D2, and carbohydrates). They are low caloric foods due to low lipids level (free fatty acids, mono-, di- and triglycerides, sterols, and phospholipids) (Heleno et al., 2009; Kalac, 2009, 2012). Beyond the nutritional characteristics, several medicinal properties have been reported, mainly due to their richness in bioactive compounds, secondary metabolites, including phenolic compounds (Carocho & Ferreira, 2013), polysaccharides (Ren et al., 2016), and lipids (Heleno et al., 2009), that present antioxidant (Ferreira et al., 2009), antitumor (Ferreira et al., 2010), anti-inflammatory (Taofiq et al., 2015), antibacterial (Alves et al., 2012) and antifungal (Alves et al., 2013) properties, among other bioactivities.

Not just fruiting bodies, also their mycelium has been recognized as a source of natural new drugs. In this context, mushroom mycelium is produced through novel biotechnologies in order to produce bioactive compounds using as pharmaceutical agent or functional food (Reis et al., 2011).

This work deals the production of mushroom mycelium for food drugs, using different solid and liquid culture media. Furthermore, to contribute to the knowledge of bioactivity of phenolic acids and ergosterol extract obtained from fruiting bodies, mycelium and culture media of two species *Pleurotus eryngii* and *Suillus bellinii*.

CHAPTER 2. INTRODUCTION

1. Studied mushroom species

1.1 *Pleurotus eryngii* (DC.) Quél.

The genus *Pleurotus* (Fries) Kummer was defined by Paul Kummer in 1871 and belongs to the phylum Basidiomycota and the order Agaricales. Many of the species of this genus produce mushroom that usually live in wood and grow in clusters. They have a range of spores varying from white to pink, brown, and nearly black (Trudell & Ammirati, 2009).



Figure 1. Fruiting body of *Pleurotus eryngii* (DC.) Quél.

This group of mushrooms can be recognized by its rather soft and fleshy fruiting body (Arora, 1986). It exhibits a short-term growth compared to other mushrooms (Liu et al., 2016), it has a high nutritional value and several therapeutic properties, being also used in different biotechnological and environmental applications (Knop et al., 2015; Corrêa et al., 2016).

Pleurotus eryngii (DC.) Quél. is a widespread species in the Mediterranean, central Europe, central Asia, and North Africa. It has a convex cap and very decurrent whitish gills (**Figure 1**). It is a weak parasite, and can live on the root or stem base of plants. *P. eryngii* taxon is described as ‘complex’ due to the significant variations in morphology, biochemical and genetic makeup so it has a wide geographical distribution. It is also used in biotechnological processes including food production, bioremediation of soil and industrial waters, biotransformation of raw plant materials to feed, among others (Stajic et al., 2009; Reis et al., 2014).

Additionally, it has a high nutritional value, so the commercial production of this species has, in the last few decades, increased rapidly worldwide. The nutritional value of *P. eryngii*

reported by Reis et al., (2014) was as follows: moisture (82.59 ± 0.36 g/100 g fw), ash (14.95 ± 0.91 g/100 g dw), proteins (2.09 ± 0.01 g/100 g dw) and energy (362.00 ± 2.06 kcal/100 g dw); its free sugars were mannitol (1.40 ± 0.09 g/100 g dw) and trehalose (14.21 ± 0.23 g/100 g dw), and tocopherols included α -tocopherol (6.79 ± 1.80 μ g/100 g dw), β -tocopherol (48.24 ± 9.53 μ g/100 g dw) and γ -tocopherol (31.55 ± 7.56 μ g/100 g dw). It presented low levels of fatty acids being the major ones palmitic acid ($17.44 \pm 0.21\%$), stearic acid ($4.77 \pm 0.08\%$), oleic acid ($47.52 \pm 0.07\%$) and linoleic acid ($24.71 \pm 0.28\%$). The percentages of saturated, monounsaturated and polyunsaturated fatty acids were $25.79 \pm 0.18\%$, $49.05 \pm 0.24\%$ and $25.17 \pm 0.24\%$, respectively (Reis et al., 2014). The main organic acids found in *P. eryngii* were oxalic acid (2.02 ± 0.03 mg/g dw), malic acid (18.48 ± 0.07 mg/g dw), citric acid (28.73 ± 0.57 mg/g dw) and fumaric acid (2.50 ± 0.05 mg/g dw) (Barros et al., 2013).

P. eryngii is esteemed primarily for its nutritional value, but it can also have bioactive properties through different active substances such as polysaccharides, lipids, peptides, sterols, dietary fibers, etc. Furthermore, several recent studies have indicated that *P. eryngii* polysaccharides have several functions, including hepatoprotector, immunity enhancer and antihyperlipidemia, and can inhibit the growth of several types of tumor cell lines (Ren et al., 2016).

1.2 *Suillus bellinii* (Inzenga) Watling

The genus *Suillus* (Inzenga) Watling is equivalent to the Boletaceae (Hawksworth et al., 1995); it is a genus from the phylum Basidiomycota and order Agaricales (Arora, 1986).

The fruiting bodies are yellow, brown and reddish in many species of the genus (Trudell & Ammirati, 2009). In addition, Arora, (1986) illustrated that the genus *Suillus* has a “slippery” cap, the pores are radially elongated in some species, having a veil or a glandular-dotted stem or both, and being mycorrhizal almost exclusively with conifers. Furthermore, *Suillus* spp. is considered C-demanding species able to produce large amounts of biomass and produce large amounts of exudates (Izumi et al., 2013).

Suillus bellinii (Inzenga) Watling is known as an ectomycorrhizal symbiont associated with a wide range of plant hosts (**Figure 2**) (Franco & Castro, 2015).



Figure 2. Fruiting body of *Suillus bellinii* (Inzenga) Watling

Suillus bellinii has different organic acids such as malic acid, quinic acid (31.2 ± 1.37 g/kg dw); 42% of nonaromatic acids; citric acid (11.2 ± 0.27 g/kg dw); 20% of nonaromatic acids and also fatty acids, mainly palmitic acid (160.702 mg/kg dw) (Ribeiro et al., 2009).

S. bellinii is also rich in alcohols, which are considered to be the main odorants of the mushroom like aroma (Guedes De Pinho et al., 2008).

2. Bioactive compound in wild mushrooms

Mushrooms have become attractive as functional foods and as a source of physiologically beneficial bioactive compounds. Herein, we will give emphasis to phenolic acids and ergosterol.

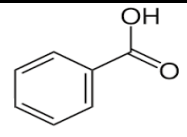
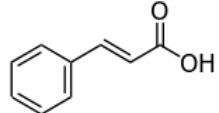
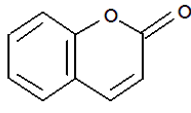
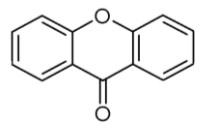
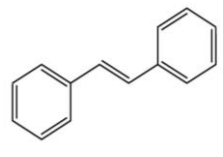
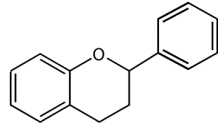
2.1 Phenolic acids

Phenolic compounds represent about 8000 different phenolic structures. They have one or more aromatic rings with one or more hydroxyl groups, including different subclasses such as flavonoids, phenolic acids, stilbenes, lignans, tannins, and oxidized polyphenols (Crozier et al., 2006; Barros et al., 2009; Fraga et al., 2010; Carocho & Ferreira, 2013). **Table 1** displays their structure as well as the number of carbons and representative compounds.

They are secondary metabolites that have an important role in health-promoting and nutraceutical potential of mushrooms in food (Chan et al., 2014; Tulio et al., 2014). The content of these compounds is highly correlated with the antioxidant activity of mushrooms and thus can be used as the important determinant in evaluating free radicals and scavenging

potential of the mushroom extracts (Chan et al., 2011; Bertalanic et al., 2012; Skotti et al., 2014).

Table1. Classification through number of carbons and basic structure of molecules within the phenolic compounds family (Carocho & Ferreira, 2013).

Number of carbons	Classification	Example	Basic structure
7	Hydroxybenzoic acids	Gallic acid	
9	Hydroxycinnamic acids	<i>p</i> -Coumaric acid	
9	Coumarins	Esculetin	
13	Xanthons	Mangiferin	
14	Stilbenes	Resveratrol	
15	Flavonoids	Naringenin	

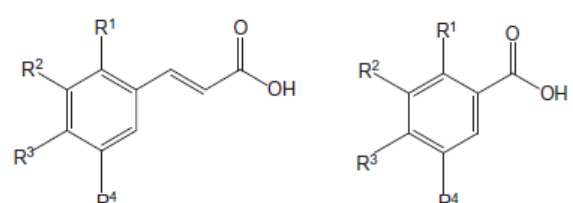
Several research studies have reported the bioactive potential of phenolic acids as the main phenolic compounds found in mushroom, as well their quantity was commonly determined by HPLC (High performance Liquid Chromatography) (Ferreira et al., 2009; Heleno et al., 2015).

Phenolic acids can be splitted into two major categories, hydroxybenzoic acids and hydroxycinnamic acids, which are derived from non-phenolic molecules of benzoic and cinnamic acid, respectively. These compounds have at least one aromatic ring in which at least one hydrogen is substituted by a hydroxyl group (**Figure 3**) (Heleno et al., 2014).

Hydroxybenzoic acid derivatives occur in the bound form and are typically a component of a complex structure like lignins and hydrolyzable tannins. They can also be found associated to

sugars or organic acids in plant foods. Hydroxycinnamic acid derivatives are present in the bound form, linked to cell-wall structural components, including cellulose, lignin, and protein, as well as linked to organic acids (Ferreira et al., 2009).

Phenolic acids are synthesized from the shikimate pathway from L-phenylalanine or L-tyrosine (Rice-Evans et al., 1996) (**Figure 4**). These amino acids are the common precursors of the majority of the phenolic natural products.



Substitution	Cinamic acid derivatives	Benzoic acid derivatives
R ¹ =OH	<i>o</i> -Coumaric acid	-
R ³ =OH	<i>p</i> - Coumaric acid	<i>p</i> - Hydroxybenzoic acid
R ³ =R ⁴ =OH	Caffeic acid	Protocatechuic acid
R ² =OCH ₃ , R ³ =OH	Ferulic acid	Vanillic acid
R ² =R ³ =OCH ₃	-	Veratric acid
R ² =R ³ =R ⁴ =OH	-	Gallic acid
R ¹ =R ⁴ =OH	-	Gentisic acid
R ² =R ⁴ =OCH ₃ , R ³ =OH	Sinapic acid	Syringic acid
R ¹ =OH, R ⁴ =HSO ₃	-	5- Sulphosalicylic acid
R ² =R ³ =OH	3,4 or 5- <i>O</i> -caffeoylquinic acid *	-

* The carboxylic group is esterified with quinic acid.

Figure 3. Chemical structures of benzoic and cinnamic acid derivatives usually found in mushrooms (Heleno et al., 2014).

Phenolic acids are abundant in a balanced daily diet, showing different bioactivities such as antioxidant (Rice-Evans et al., 1996, Ferreira et al., 2009), antitumor (Carocho & Ferreira, 2013), and antimicrobial (Alves et al., 2013a) properties. Cinnamic acid has strong capacity to inhibit the growth of human tumor cell lines such as non-small lung carcinoma (NCI-H460) (Vaz et al., 2012), colon carcinoma (HCT15) and cervical carcinoma (HeLa) cell lines (Heleno et al., 2014a, 2015).

Mushrooms have been extensively studied due to their bioactive properties (Mau et al., 2002; Murcia et al., 2002; Yang et al., 2002; Ferreira et al., 2009) assigned to several molecules including phenolic acids (Valentão et al., 2005; Puttaraju et al., 2006; Ribeiro et al., 2007; Kim et al., 2008; Barros et al., 2009). Nevertheless, *in vivo*, these compounds are metabolized and circulate in the organism as glucuronated, sulphated and methylated metabolites (Heleno et al., 2014).

The main phenolic acids reported in *P. eryngii* are *p*-hydroxybenzoic acid (3.81 ± 0.41 mg/100 g dw) (Reis et al., 2014), gallic acid and protocatechuic acid (Ferreira et al., 2009; Heleno et al., 2015).

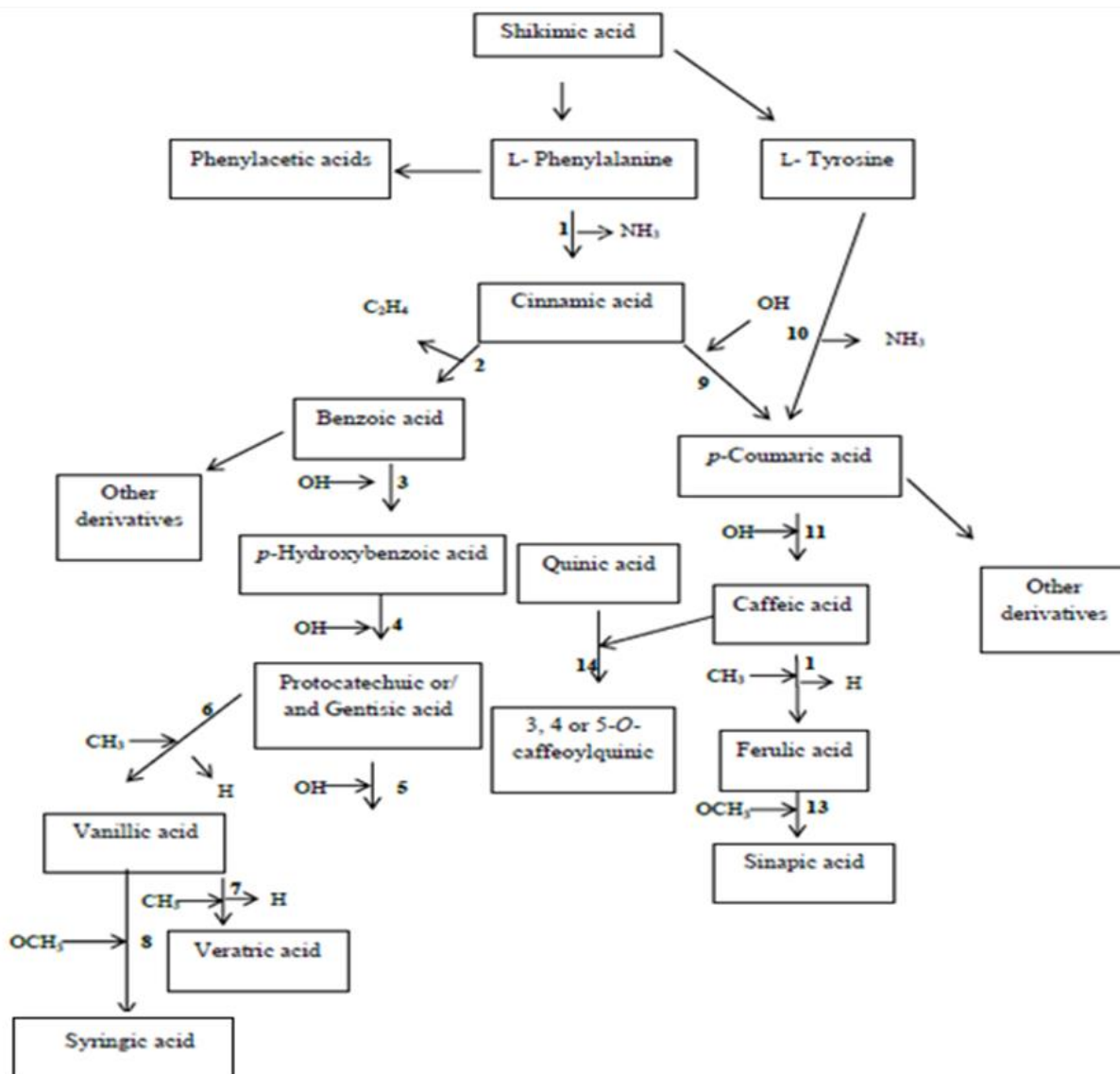
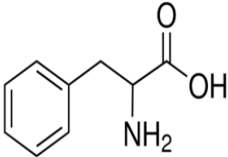
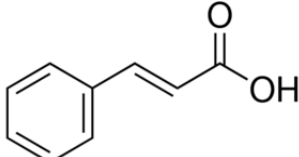
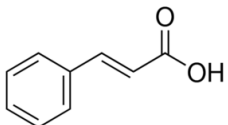
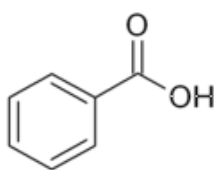
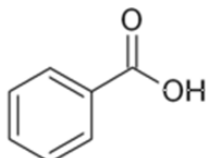
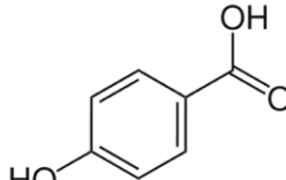
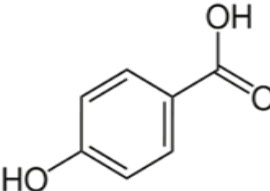
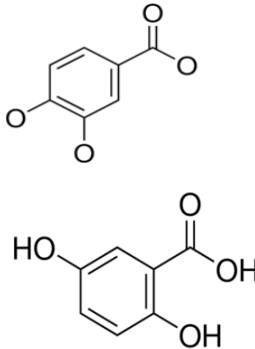
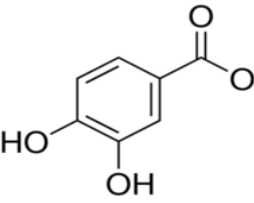
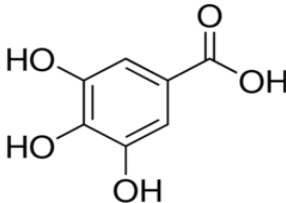
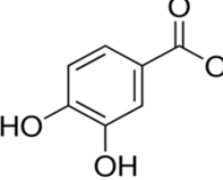
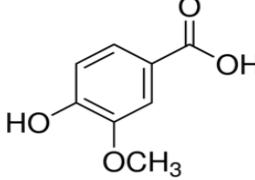
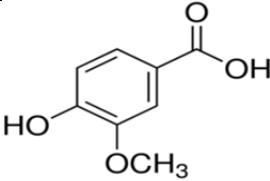
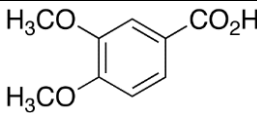
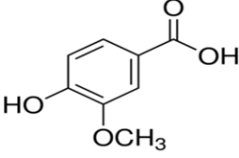
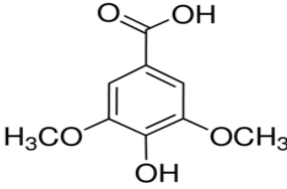
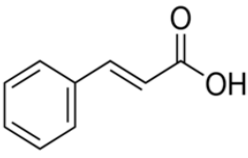
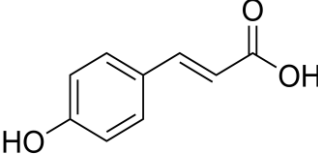
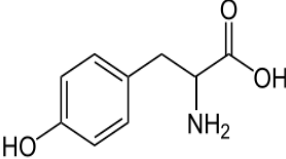
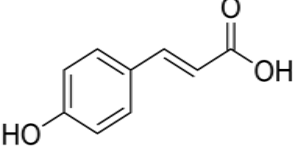
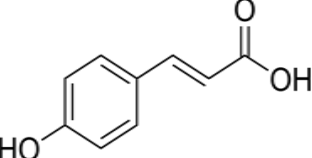
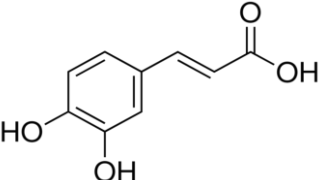
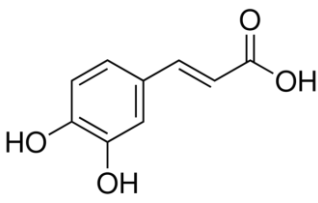
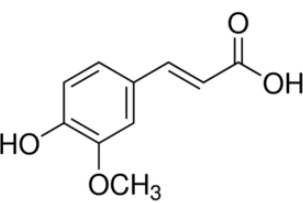
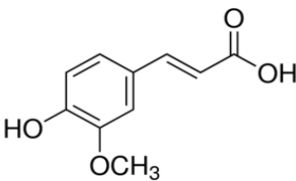
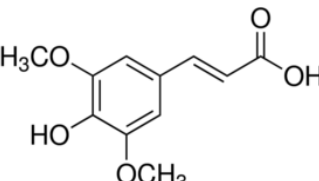


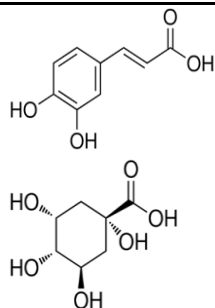
Figure 4. General representation of the phenolic acids biosynthesis derived from the shikimic acid pathway. (Heleno et al., 2014). The numbers find the correspondence in the entries of **Table 2**.

Table 2. Main enzymes involved in the biosynthesis of phenolic acids through shikimate pathway from L-phenylalanine or L-tyrosine.

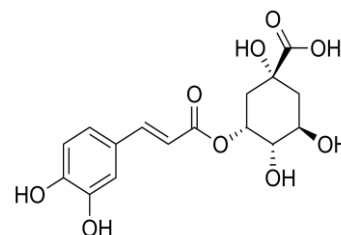
Entry	Starting molecule	Enzyme	Final compound
1	 Phenylalanine	Phenylalanine ammonialyase (PAL)	 Cinnamic acid
2	 Cinnamic acid	Oxidase (presumed b-oxidation)	 Benzoic acid
3	 Benzoic acid	Benzoic acid 4-hydroxylase	 <i>p</i> -Hydroxybenzoic acid
4	 <i>p</i> -Hydroxybenzoic acid	<i>p</i> -Hydroxybenzoic acid 3-hydroxylase	 Gentisic acid
5	 Protocatechuic acid	Protocatechuic acid 5-hydroxylase	 Gallic acid
6	 Protocatechuic acid	Protocatechuic acid 3-O-methyltransferase	 Vanillic acid

7	 <p>Vanillic acid</p>	Vanillic acid 4-O-methyltransferase	 <p>Veratric acid</p>
8	 <p>Vanillic acid</p>	Vanillic acid 5 hydroxylase and vanillic acid 5-O-methyltransferase	 <p>Syringic acid</p>
9	 <p>Cinnamic acid</p>	Cinnamic acid 4-hydroxylase	 <p><i>p</i>-Coumaric acid</p>
10	 <p>L- Tyrosine</p>	Tyrosine ammonia lyase (TAL)	 <p><i>p</i>-Coumaric acid</p>
11	 <p><i>p</i>-Coumaric acid</p>	<i>p</i> -Coumaric acid 3-hydroxylase	 <p>Caffeic acid</p>
12	 <p>Caffeic acid</p>	Caffeic acid 3-O-methyltransferase	 <p>Ferulic acid</p>
13	 <p>Ferulic acid</p>	Ferulic acid 5-hydroxylase and caffeic/5-hydroxyferulic acid O-methyltransferase (COMT)	 <p>Sinapic acid</p>

14



Caffeic + quinic acid

4-Caffeate CoA ligase and
quinic acid hydroxycinnamoyl
transferase

3,4 or 5-O-caffeoylquinic acid

2.2 Ergosterol

The presence of sterols in animals, plants and fungi has been reported by Barreira & Ferreira, (2015). They are organic compounds with a tetracyclic structure of four rings linked together, of three to six carbons and other ring with five carbons (steroid nucleus) (**Figure 5**) with a hydroxyl group at the C3 position and an aliphatic chain linked to the steroid nucleus (Fahy et al., 2005).

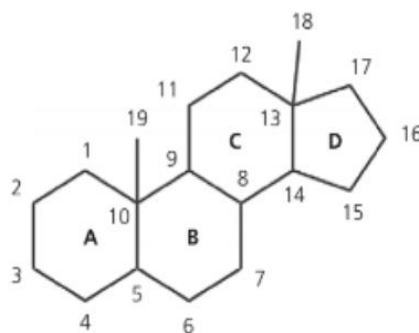


Figure 5 . Steroid nucleus.

Ergosterol is evidently the main sterol found in mushrooms, being a precursor of vitamin D2 (**Figure 6**) (Mattila et al., 2002; Barreira & Ferreira, 2015).

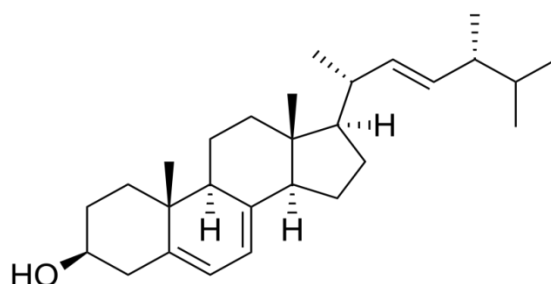


Figure 6. Chemical structure of ergosterol.

Ergosterol is a solid compound, crystalline, colourless with the scientific name 5,7,22-ergostatien-3 β -ol, the empirical formula C₂₈H₄₄O and a molecular weight of 396.36 g/mol. Its melting point is in the order of 161-166°C. In addition, ergosterol supports the temperature of 250 °C without decomposition. It has a side chain with a double bond at C22 and two double bonds at the level of positions C5 and C7 (ring B), giving a maximum absorption in the UV in a range of 240 to 300 nm (Barreira et al., 2014). Ergosterol is practically insoluble in water; however, it is soluble in organic solvents, such as chloroform. Exposure to ultraviolet light causes a photochemical reaction that converts ergosterol to ergocalciferol (vitamin D₂) (Villares et al., 2012).

According to Barreira et al., (2014), ergosterol can be determined by different analytical methods such as high performance liquid chromatography coupled to ultraviolet detection. Savón et al., (2002) and Jasinghe & Perera, (2005) reported that the concentration of ergosterol in mushrooms, depends on the part of the mushroom tissue, developmental stage, growth conditions, and environmental factors.

Sterols, in general, have been reported to play several biological functions that include antioxidant (Shao et al., 2010), anti-inflammatory (Kuo et al., 2011) and antitumoral activity (Villares et al., 2012; Barreira & Ferreira, 2015), having also the ability to activate the expression of specific defense genes (Lochman & Mikes, 2005). Furthermore, these compounds permit a reduction in pain related inflammation, reduce cardiovascular disease and inhibit cyclooxygenase (COX) enzymes (Yasukawa et al., 1994; Zhang et al., 2002; Ravi & Abplanalp, 2003).

The ergosterol content in *Pleurotus eryngii* obtained after Soxhlet extraction with hexane and a saponification step was 187 \pm 1 mg/100 g dw (Barreira et al., 2014).

3. Bioactivity of wild mushroom extracts

Mushrooms grow in darkness and dampness in highly competitive environments and protect themselves from microbes by excretion of natural substances; this explains their richness in bioactive compounds (Ferreira et al., 2010).

In fact, mushrooms have been considered to have excellent nutritional attributes (Kalac, 2009). Their medicinal properties have been studied which include antioxidant (Puttaraju et al., 2006; Ferreira et al., 2009; Heleno et al., 2015), antitumor (Moradali et al., 2007; Ferreira et al., 2010; Caroch & Ferreira, 2013), antimicrobial (Alves et al., 2012, 2013),

immunomodulator (Borchers et al., 2008), antiatherogenic (Mori et al., 2008), hypoglycemic (Hu et al., 2006) and anti-inflammatory (Taofiq et al., 2016) activities.

3.1 Antioxidant activity

Oxidation is essential to many organisms, which can produce energy to fuel biological processes (Huanga et al., 2015).

A free radical is an atom or molecule possessing unpaired electrons in the outer orbit, generally unstable and very reactive (Halliwell & Gutteridge, 1999; Gutteridge & Halliwell, 2000; Carocho & Ferreira, 2013a). Free radicals and non-radical species are produced in the normal metabolism of aerobic cells, mostly in the form of oxygen or/and nitrogen reactive species (ROS or/and RNS) (Valko et al., 2007; Ferreira et al., 2009). They can be produced in the mitochondria, through xanthine oxidase, peroxisomes, inflammation processes, and phagocytosis, among other. Nevertheless, some external factors also help to promote the production of free radicals such as smoking, environmental pollutants, radiation, drugs, pesticides, industrial solvents and ozone (**Figure 7**) (Ferreira et al., 2009).

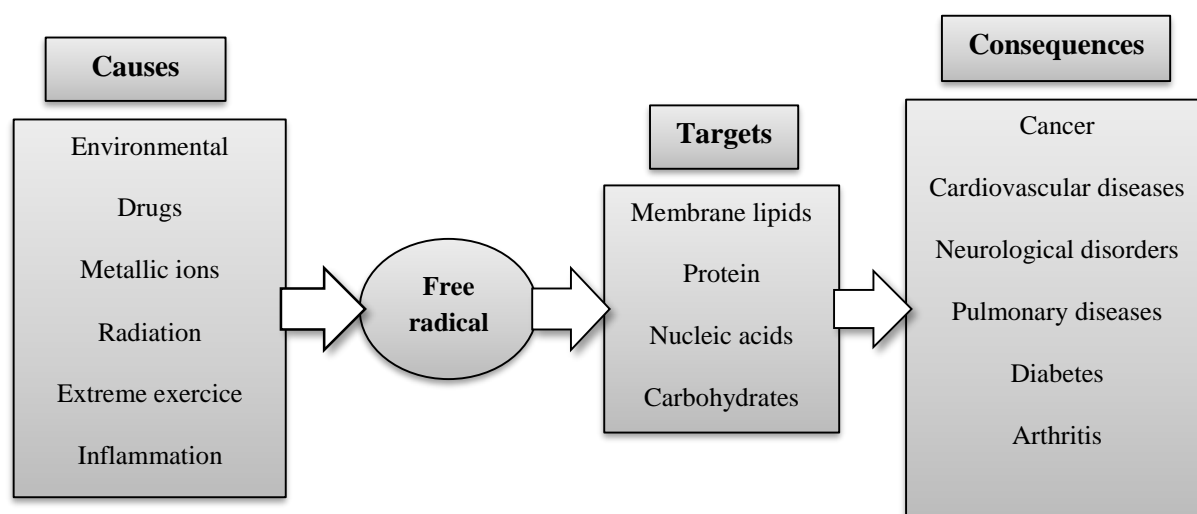


Figure 7. Major causes for over production of free radical (oxidative stress), possible cellular targets and conditions that were associated to oxidative stress (Ferreira et al., 2009).

Free radicals are neutralized by cellular antioxidant defenses such as enzymes and non-enzymatic molecules. Maintaining the equilibrium between free radicals production and antioxidant defenses is an essential condition for normal organism functioning; this equilibrium might be displaced either by the overproduction of ROS or by the loss of the cell antioxidant defenses (**Figure 8**) (Ferreira et al., 2009; Lobo et al., 2010; Carocho & Ferreira, 2013a). When free radicals are in excess, the organism is in oxidative stress, and this situation

leads to oxidation and damage of cellular lipids, proteins and DNA, and causing several diseases including cancer, cardiovascular diseases, neurological disorders, renal disorders, liver disorders, hypertension, among others (Valko et al., 2007; Ferreira et al., 2009; Lobo et al., 2010, Carocho & Ferreira, 2013a). Several ROS production pathways and the main endogenous antioxidant defenses of the cell are described in **Figure 9** (Ferreira et al., 2009).

Moreover, the synthetic antioxidants most commonly used in industrial processing may be cytotoxic. Thus, it is necessary to enhance natural and nontoxic antioxidants (Yating et al., 2015), that can be found in mushrooms.

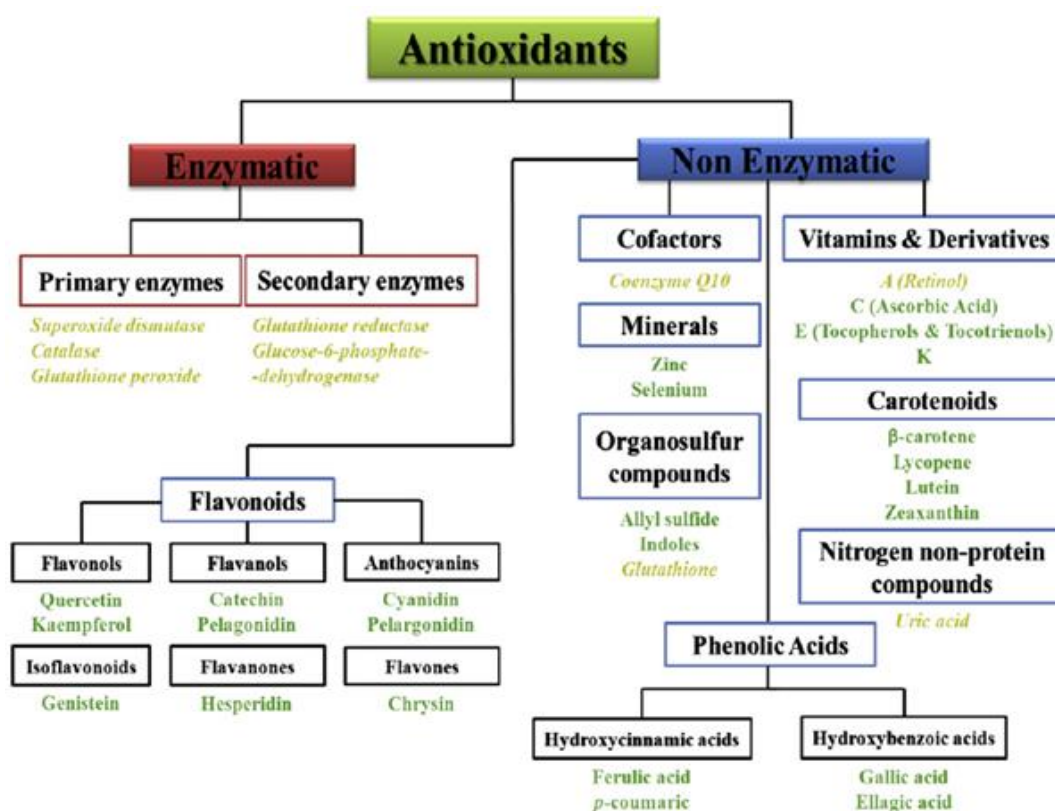


Figure 8. Natural antioxidants separated in classes. Green words represent exogenous antioxidants, while yellow ones represent endogenous antioxidants (Carocho & Ferreira, 2013a).

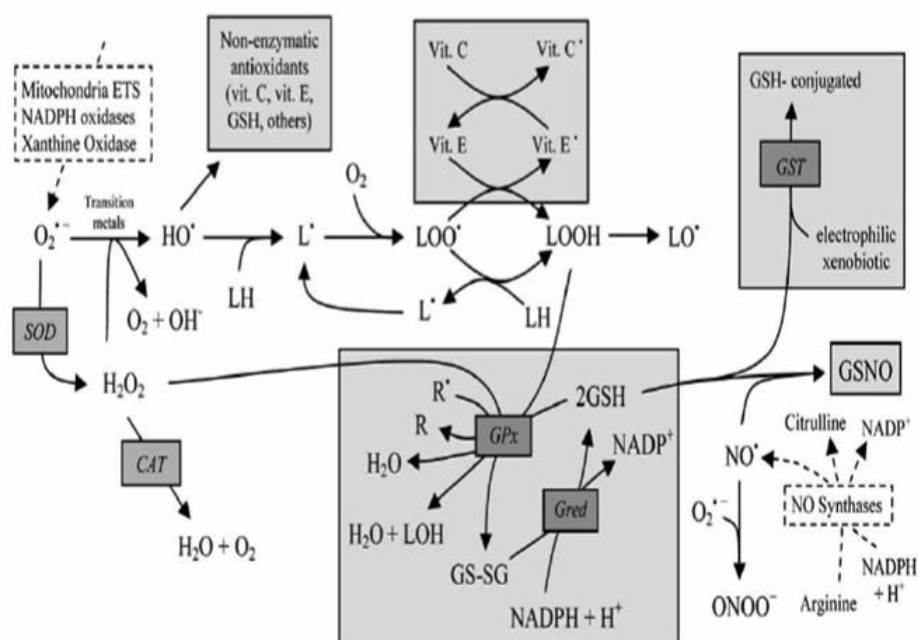


Figure 9. Overview of the main reactions involving reactive Oxygen species (ROS) / reactive Nitrogen species (RNS), and major endogenous enzymatic and non-enzymatic antioxidant defenses in the cell. The most representative endogenous sources (traced rectangles) of ROS/RNS are presented and include: Mitochondrial ETS (Electron transport system), NADPH oxidases, Xanthine oxidase for ROS and NO synthases for RNS. The main antioxidant defenses are presented in shaded rectangles and the enzymes involved are presented in *italics*. Molecular Oxygen (O_2), superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (HO^{\cdot}), hydroxide ion (HO^-), membrane lipids (LH), lipid radical (L^{\cdot}), peroxyl radical (LOO^{\cdot}), hydroperoxide lipid ($LOOH$), lipid alkoxyl radical (LO^{\cdot}), nitric oxide (NO^{\cdot}), radicals (R^{\cdot}), non-radicals (R), alcohols (LOH), glutathione (GSH), glutathione disulphide (GS-SG), α -tocopherol or vitamin E (vit. E), vitamin E radical (vit. E $^{\cdot}$), vitamin C (vit. C), vitamin C radical (vit. C $^{\cdot}$), S-nitrosoglutathione (GSNO), nicotinamide adenine dinucleotide phosphate: oxidized ($NADP^+$), reduced ($NADPH$). Enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (Gred), glutathione- S-transferases (GST), Mitochondrial ETS (electron transport system), nitric oxide synthase (NOS) (Ferreira et al., 2009).

In this context, mushrooms contain many different compounds with powerful antioxidant activities, such as phenolic compounds and vitamins (Ferreira et al., 2009) that help the organism in the combat against oxidative stress. As an example, **Table 3** shows antioxidant effects in extracts prepared from the fruiting bodies of the species studied within the present work.

Particularly, *P. eyngii* showed high reducing power and free radical-scavenging activity (Reis et al., 2014).

Table 3. Antioxidant activity of methanolic extracts prepared from fruiting bodies and mycelia of the species studied within this work.

Species	Part	Antioxidant assay	
<i>Pleurotus eryngii</i> (Reis et al., 2012)	Fruiting body	Folin Ciocalteu assay mg (GAE/g extract)	7.14 ± 2.01
	Mycelium (MMN)		9.11 ± 0.23
	Fruiting body	Ferricyanide/Prussian blue assay; EC ₅₀ (mg/mL)	3.72 ± 0.09
	Mycelium (MMN)		3.81 ± 0.02
	Fruiting body	DPPH scavenging activity assay; EC ₅₀ (mg/mL)	8.67 ± 0.12
	Mycelium (MMN)		25.40 ± 0.33
	Fruiting body	β-carotene/linoleate assay; EC ₅₀ (mg/mL)	4.68 ± 0.60
	Mycelium (MMN)		1.43 ± 0.60
	Fruiting body	TBARS assay; EC ₅₀ (mg/mL)	3.95 ± 0.58
	Mycelium (MMN)		21.03 ± 0.45
<i>Suillus bellinii</i> (Ribeiro et al., 2006)	Fruiting body	DPPH scavenging activity assay: At 150 μg/mL, 35% of scavenging activity.	

DPPH: 2,2-Diphenyl-1-picrylhydrazyl; TBARS: thiobarbituric acid reactive substances; EC₅₀: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance for the Ferricyanide/Prussian blue assay. GAE: Gallic acid equivalents.

3.2 Anti-inflammatory activity

Inflammation is a biological response to eliminate the initial cause of cell injury or harmful toxins stimuli such as pathogens, damaged cells, or irritation. However, inflammation may lead to many diseases including atherosclerosis, obesity, metabolic syndrome, diabetes and cancer (Moro et al., 2012; Taofiq et al., 2016). Macrophages are important components of the immune system, and they play a crucial role in the inflammatory response by supplying an immediate defense against foreign agents and release of excess pro-inflammatory mediators such as interleukins (IL-1β, IL-6, IL-8), tumor necrosis factor (TNF-α), nuclear factor-κB (NF-κB), intercellular adhesion molecule-1 (ICAM-1), inducible type cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), 5-lipoxygenase (5-LOX), and inducible nitric oxide synthase (iNOS) that leads to the production of reactive nitrogen species such as nitric oxide (NO). Overproduction of these inflammatory mediators leads to different kinds of cell damage (Joo et al., 2014; Taofiq et al., 2015).

Therefore, pharmacologic treatment with anti-inflammatories generally begins with nonsteroidal anti-inflammatory drugs (NSAIDs). In contrast, the long-term administration of NSAIDs is associated with a poor safety profile on gastrointestinal, cardiovascular, renal, also high blood pressure and acute tubular necrosis (Taofiq et al., 2015; Rovati et al., 2016).

In this case, discovering of natural bioactive compounds with anti-inflammatory properties and without or lower toxic impact is essential. Mushrooms have also demonstrated some anti-inflammatory potential based on their ability to reduce the production of inflammatory mediators (Taofiq et al., 2016).

Previous research studies have been carried out on several mushroom species, mainly in methanolic and ethanolic extracts (**Table 4**).

Table 4. Anti-inflammatory activity of extracts prepared from fruiting bodies of the species studied within this work.

Species	Extract	NO production inhibition
<i>Pleurotus eryngii</i>	Ethanolic	EC ₅₀ value: 388 ± 17 µg/mL (Taofiq et al., 2015).
<i>Suillus bellinii</i>	Not available in literature.	

EC₅₀: Extract concentration corresponding to 50% of NO production inhibition.

3.3 Cytotoxic activity

Carcinogenesis is one of the most life-threatening diseases and causes severe health problems in both developed and developing countries. It is a process which normally takes several years during which progressive genetic changes occurs leading to malignant transformations (Ferreira et al., 2010; Jemal et al., 2011; Carocho & Ferreira, 2013; Rashed et al., 2014).

Several endogenous causes are known to increase the risk of cancer. They include external factors such as smoking, dietary factors, infections, exposure to radiation, lack of physical activity, obesity, and environmental pollutants (Anand et al., 2008), and internal factors such as chromosomal abnormalities, somatic mutations, and immunological surveillance (Carocho & Ferreira, 2013a).

Furthermore, literature suggests that there is a link between cancer and oxidative stress. Therefore, the consumption of foods with antioxidant activity may reduce oxidative stress associated to these diseases (Vaz et al., 2010).

To prevent the growing understanding of the molecular biology of cancer, chemotherapy is used as therapeutic modalities for the treatment of cancer, but most of the anticancer drugs applied in chemotherapy are cytotoxic to normal cells. Consequently, searching for natural anticancer drugs with low toxicity and high efficacy becomes very important (Ren et al., 2016).

In this context, mushrooms are a vast source of powerful potential pharmaceutical drugs. They have been established to inhibit the growth of different tumor cell lines (Poucheret et al., 2006). Moreover, both cellular components and secondary metabolites of a large number of mushrooms have anticarcinogenic actions including, low-molecular-weight (LMW), and high-molecular-weight compounds (HMW) (**Figure 10**) (Wasser & Weis, 1999; Ferreira et al., 2010).

Mushrooms have powerful capacities against cancers of the stomach, esophagus, and lungs, among others and are known in China, Japan, Korea, Russia, United States and Canada (Ferreira et al., 2010).

The most important molecules found in mushrooms with antitumor potential are polysaccharides and phenolic compounds or derivatives (Vaz et al., 2012).

Several recent studies have indicated that *P. eryngii* polysaccharides can inhibit the growth of several types of cancer (Ren et al., 2016) (**Table 5**).

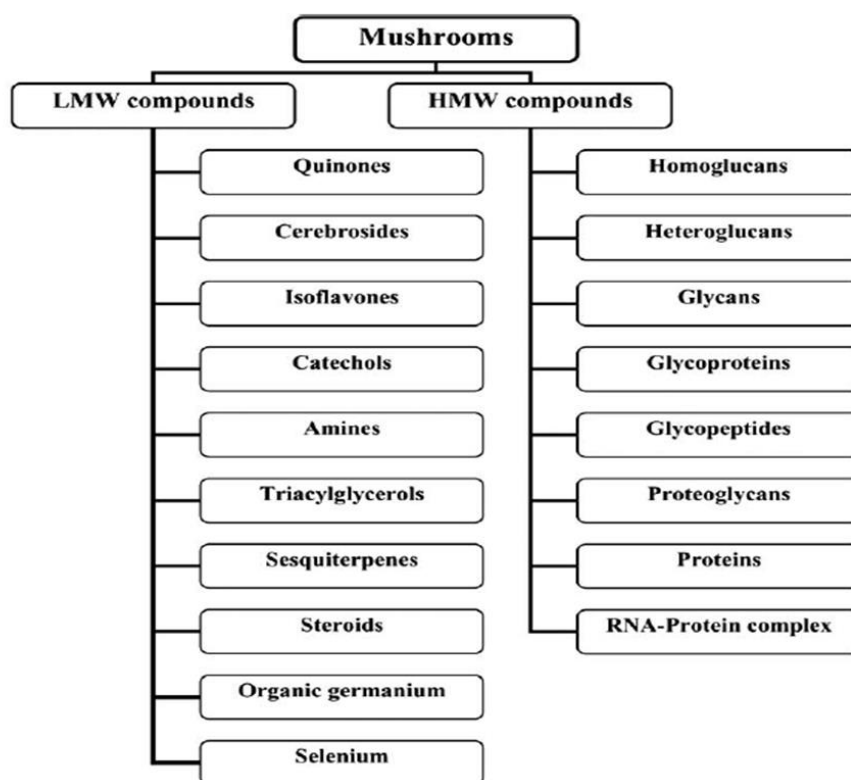


Figure 10. Low-molecular-weight (LMW) and high-molecular-weight (HMW) compounds with antitumor potential found in mushrooms (Ferreira et al., 2010).

Table 5. Cytotoxic activity of extracts prepared from fruiting bodies of the species studied within this work.

Species	Extract	Type of activity
<i>Pleurotus eryngii</i>	Polysaccharidic	Inhibited HepG-2 proliferation, induced apoptosis, cell cycle arrest at the S-phase and intracellular ROS production (Ren et al., 2016).
<i>Suillus bellinii</i>		Not available in literature.

4. Production of mushroom mycelia by *in vitro* culture

4.1 *In vitro* culture applied to mushrooms

The filamentous fungi are characterized by being non-mobile organisms composed of an organized mass of threadlike cells called hyphae (the assembly of hyphae are called mycelium), having a life cycle with sexual and asexual reproduction that results in spores production, usually from a common haploid "stem" resulting from zygotic meiosis and heterotrophic nutrition (Griffin, 1994; Alexopoulos et al., 1996).

Leiva et al., (2015) pointed out that mycelium growth is affected by the *in vitro* conditions, such as size of the inoculum, the period of incubation, pH, temperature, composition of media, thus, cultures on agar and in liquid media are excellent models and are responsible for mycelium development. PDA (Potato Dextrose Agar) and MMN (Melin-Norkrans medium) have been the successfully media for the cultivation of many wild mushrooms as demonstrated by Pinto et al., (2013).

Stamets and Chilton, (1983) explain the methodology of culture in solid medium: "Cut a piece of the interior cap tissue or the upper region of the "stem" from mushroom and put the fragment at the center of a Petri dish. This technique takes place in a sterile area to avoid contamination (yeast, bacteria), that would stop the growth of mycelia".

In vitro culture methodology (for mycelia production) is used as alternative products of fruiting bodies, containing numerous advantages including the shorter cultivation time, storage of mycelia for a long period without genetic alterations, contamination can be easily observed and monitored on the flat of media, so it is easy to know and retain pure culture, it is also important for the preservation of endangered or rare species (Stamets & Chilton, 1983; Ferreira et al., 2009; Zilly et al., 2011; Heleno et al., 2012; Pinto et al., 2013). In addition to these advantages, *in vitro* culture allows the scaling up of biomass production for biotechnological purposes. Not only fruiting bodies, but also mycelia are a source of nutraceuticals and functional foods. Extraction and purification of bioactive compounds such

as polysaccharides (Chen et al., 2013), phenolic compounds (Reis et al., 2012) and some other from mycelium it's easy, and could be isolated for medicinal purposes (Pinto et al., 2013), including antioxidant, anti-inflammatory, antitumor, antimicrobial, antiviral, antidiabetic, immunomodulatory, cardiovascular, liver protective, and antifibrotic properties (Borchers et al., 2004; Gonçalves et al., 2011). In this context, Reis et al., (2012) was the first publication comparing *in vivo* and *in vitro* culture in terms of bioactivity.

Different media influence growth rates and mycelia biomass production, since liquid MMN media contains available nutrients and oxygen leading to higher mycelia absorption of nutrients, thus a faster growth and higher mycelia biomass (Heleno et al., 2012). On other hand, culture media also may influence the bioactivity of mycelium. Heleno et al., (2012) demonstrated that PDA is the most indicated medium to increase the antioxidant activity of *G. lucidum* mycelium.

Corrêa et al., (2016) in a recent review reported that *Pleurotus* spp mycelium has high nutritional profile that determines a precious aroma, characteristic flavor, and pharmacological value being a source of important bioactive compounds such as peptides, glycoproteins, polysaccharides, lipids and hydrolytic and oxidative enzymes with antioxidant, antimicrobial, anti-inflammatory, antitumor and immunomodulatory effects.

4.2 Case-studies with wild mushrooms

The antioxidant properties of wild mushrooms with their content in antioxidant compounds including tocopherols, can detoxify damaging forms of activated oxygen. A comparative study of tocopherols composition and antioxidant properties of *in vivo* (fruiting bodies) and *in vitro* (mycelia) ectomycorrhizal fungi: *Paxillus involutus* and *Pisolithus arhizus* was carried out by Reis et al., (2011). Mycelia showed higher levels of total tocopherols than fruiting bodies, and particularly *P. arhizus* mycelium demonstrated to be an efficient source of γ -tocopherol.

A systematic study was performed in order to compare the antioxidant activity of phenolic and polysaccharidic extracts from fruiting body, spores and mycelium, obtained in three different culture media, of *Ganoderma lucidum*. The highest levels of total polysaccharides (~14 mg/g extract) and individual sugars were detected in mycelia collected from solid culture media (Heleno et al., 2012).

Another study examines a comparison of the antioxidant activities and phenolic profile of cultivated mushrooms and their mycelia: *Agaricus bisporus*, *Pleurotus ostreatus*, *Pleurotus eryngii* and *Lentinula edodes*. *L. edodes* mycelia have the highest reducing power. Generally, *in vivo* samples detected higher antioxidant activities than their mycelia obtained by *in vitro* culture. On the other hand, phenolic compounds were revealed both in mushrooms and mycelia without any particular abundance. Results demonstrated that there is no correlation between mushrooms and their mycelia obtained *in vitro*, thus *in vitro* production may be used as a source of bioactive compounds (Reis et al., 2012).

5. Objectives

The main goals of the present work were to produce, chemically characterize and evaluate the bioactivity of mycelia of two mushrooms species, *Pleurotus eryngii* and *Suillus bellinii*, obtained by *in vitro* culture, using different solid and liquid culture media: iMMN (incomplete Melin-Norkans medium) and PDA/PDB (Potato Dextrose Agar/ Potato Dextrose broth media).

Furthermore, the mycelia produced, their fruiting body and the culture media were:

- i) Chemically characterized in terms of phenolic acids and ergosterol by HPLC-DAD and HPLC-UV, respectively;
- ii) Evaluated regarding antioxidant (free radicals scavenging activity, reducing power and lipid peroxidation inhibition), anti-inflammatory (NO production inhibition in mice macrophages) and cytotoxic (in human tumor cell lines and in a non-tumor porcine primary liver cell culture) properties.

CHAPTER 3. MATERIALS AND METHODS

1. Wild samples and *in vitro* production of mycelia

Two species of wild mushrooms (*Pleurotus eryngii* (DC.) Quél. and *Suillus bellinii* (Inzenga) Watling) were collected in Bragança (Northeast Portugal) during November 2015. Mycelium was isolated from sporocarps of each sample on different solid: i) potato dextrose agar medium (PDA) (Biolab); ii) Melin-Norkans incomplete medium (without micronutrients, casaminoacids and malt extract) (iMMN solid), and liquid: i) potato dextrose broth (PDB); ii) Melin-Norkans incomplete (without micronutrients, casaminoacids and malt extract) (iMMN liquid) culture media (Marx, 1969).

Mycelia were grown in Petri dishes with 10 mL of solid media and flasks with 20 mL of liquid media. Petri dishes and flasks were placed at 22 °C in the dark until mycelium covered most of the medium: 21 days for *P. eryngii* and 42 days for *S. bellinii*, approximately. Radial growth measurements were registered every week from the inoculation time until the full growth of the mycelium (covering all available area). The mycelia were further recovered from the medium.

Fruiting bodies, mycelia and culture media were lyophilized (FreeZone 4.5, Labconco, MO, USA), ground to a fine powder (20 mesh) and weighted to obtain the dry biomass (dw).

2. Standards and reagents

The solvents acetonitrile 99.9% and methanol were of high-performance liquid chromatography (HPLC) grade from Lab-Scan (Lisbon, Portugal). Ergosterol, phenolic compounds (gallic, protocatechuic; *p*-hydroxybenzoic, *p*-coumaric, and cinnamic acids) and trolox (6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid) standards were purchased from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Dulbecco's modified Eagle's medium, hank's balanced salt solution (HBSS), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA, penicillin/streptomycin solution (100 U/mL and 100 mg/ mL, respectively) were purchased from Gibco Invitrogen Life Technologies (Paisley, UK). Sulforhodamine B, trypan blue, trichloroacetic acid (TCA) and Tris were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). RAW264.7 cells were purchased from ECACC ("European Collection of Animal Cell Culture") (Salisbury, UK), lipopolysaccharide (LPS) from Sigma and DMEM medium from HyClone. The Griess Reagent System Kit was purchased from Promega, and

dexamethasone from Sigma. Thiamine, casamino acids, malt extract and agar were obtained from Panreac AppliChem (Barcelona, Spain). PDA and PDB were acquired from Oxoid microbiology products (Hampshire, United Kingdom). All other reagents and solvents were of analytical grade and obtained from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

3. Preparation of the extracts

The extraction was carried out by stirring the samples (≈ 2 g) with methanol (30 mL) at 25°C and 150 rpm, for 1 h. The extract was separated from the residue by filtration through Whatman No. 4 paper to a round flask. The residue was re-extracted once more and the filtrates were combined and concentrated under vacuum (rotary evaporator, Büchi, Flawil Switzerland) (**figure 11**) (Reis et al., 2012).



Figure 11. Step of solvent removal in the preparation of the extracts.

4. Chemical characterization of the extracts

4.1 Analysis of phenolic acids

The final extracts were dissolved in methanol:water 20:80 (v/v) at 20 mg/mL and filtered through a 0.22 μ m nylon disposable filter. The analysis was performed using a Shimadzu 20A series ultra-fast liquid chromatograph (UFLC, Shimadzu Cooperation, Kyoto, Japan) as previously described by Reis et al., (2012) (**figure 12**). Separation was achieved on a Waters Spherisorb S3 ODS2 C₁₈ column (3 μ m, 150 mm x 4.6 mm) thermostatted at 35°C. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was 10% B to 15% B over 5 min, 15–25% B over 5 min, 25–35% 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. Detection was carried out in a photodiode array detector (PDA), using 280 nm as the preferred wavelength.

The phenolic acids were quantified by comparison of the area of their peaks recorded at 280 nm with calibration curves (5-100 µg/mL) obtained from commercial standards of each compound:

Protocatechuic acid ($y = 164741x$, $R^2=0.9996$), *p*-hydroxybenzoic acid ($y = 113523x$, $R^2=0.9993$), *p*-coumaric acid ($y = 433521x$, $R^2=0.9981$) and cinnamic acid ($y = 583527x$, $R^2=0.9961$). The results were expressed as µg per g of extract.



Figure 12. HPLC-DAD equipment used in the analysis of phenolic acids.

4.2 Analysis of ergosterol

The final extracts were dissolved in methanol at 20 mg/mL and filtered through a 0.22 µm nylon disposable filter. Ergosterol analysis was performed by high performance liquid chromatography coupled to an ultraviolet detector (HPLC-UV) as previously described by Barreira et al., (2014) (**Figure 13**). The components of the HPLC-UV integrated system include a pump (Knauer, Smartline system1000, Berlin, Germany), an UV detector (Knauer Smartline 2500), a degasser system (Smartline manager 5000) and an injector (autosampler) (AS-2057 Jasco, Easton, MD, USA). Chromatographic separation was performed with an Inertsil 100A ODS-3 reversed-phase column (4.6×150 mm, 5 µm BGB Analytik AG, Boeckten, Switzerland) at 35 °C (7971R Grace oven). The mobile phase was acetonitrile/methanol (70:30, v/v), at a flow rate of 1 mL/min, and the injection volume was 20 µL. The detection was performed at 285 nm and data were analysed using Clarity 2.4 Software (DataApex). Ergosterol was quantified by comparing the area of its peak with the calibration curve obtained from a commercial standard. Quantification was performed using the internal standard method and cholecalciferol was used as internal standard. The results were expressed in mg per g of extract.



Figure 13. HPLC-UV system used in the analysis of ergosterol.

5. Evaluation of bioactive properties

5.1 Antioxidant activity

The final extracts were dissolved in methanol at appropriate concentrations (10-80 mg/mL) and several dilutions were obtained from the stock solutions: 0.005-50 mg/mL, depending on the assay.

The *in vitro* antioxidant activity of the extracts was evaluated by four different assays: DPPH radical-scavenging activity, reducing power, β -carotene bleaching inhibition and thiobarbituric acid reactive substances (TBARS) assay (Heleno et al., 2010). Trolox was used as positive control.

5.1.1 DPPH radical scavenging activity

In this assay, the deep violet chromogen DPPH radical is reduced to slight yellow color in the presence of hydrogen donating antioxidants leading to the formation of non-radical form. The reaction mixture consisted of one of the different concentrations of the diluted methanolic extracts (30 μ L) and methanolic solution (270 μ L) containing DPPH radicals (6×10^{-5} mol/L). The microplate was stored in the dark at 25 °C for 1h. Absorbances were recorded using ELX800 microplate Reader (Bio-Tek Instruments, Inc.; Winooski, VT, USA) at 515 nm (**Figure 14**).

The results were expressed in mg/mL as the extract concentration responsible for 50% of DPPH radical scavenging activity (EC_{50}), calculated by interpolation from the graph of the

radical scavenging activity (RSA) percentage against extract concentration and expressed in mg/mL of extract.

$\%RSA = [(A_{DPPH} - A_s) / A_{DPPH}] \times 100$, (A_s - absorbance of the solution when the sample extract has been added at a particular level and A_{DPPH} - absorbance of the DPPH solution).

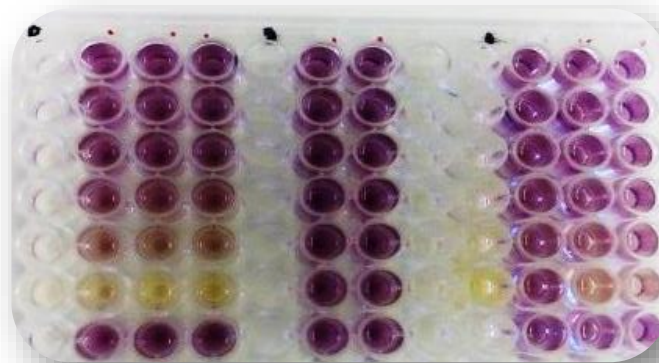


Figure 14. Microplate used in the evaluation of DPPH radical-scavenging activity.

5.1.2 Reducing power

This methodology is based on the ability to reduce yellow ferric form (Fe^{3+}) to blue ferrous form (Fe^{2+}) by the action of electron-donating antioxidants (Benzie et al., 1999).

Different concentrations of the methanolic extracts (0.5 mL), sodium phosphate buffer (0.5 mL, 200 mmol/L, pH 6.6) and potassium ferricyanide (0.5 mL, 1% w/v) were mixed in eppendorf tubes. The tubes were incubated at room temperature (50 °C) for 20 min. After the addition of trichloroacetic acid (0.5 mL, 10% w/v), the mixture (0.8 mL) was transferred into 48 well microplates as also deionized water (0.8 mL) and ferric chloride (0.16 mL, 0.1% w/v). Absorbance was then read at 690 nm in the microplate reader mentioned above (**Figure 15**). The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance against extract concentration and expressed in mg/mL of extract.

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Figure 15. Microplate used in the evaluation of reducing power.

5.1.3 Inhibition of β -carotene bleaching

This assay is based on the capacity of antioxidants to neutralize the linoleate free radical. This neutralization is detected by the discoloration of the yellowish color of β -carotene.

The reagent is prepared by dissolving 2 mg of β -carotene in 10 mL of chloroform in a round-bottom flask. This solution was concentrated in a rotary evaporator to remove the chloroform, and then 400 mg of Tween 80 emulsifier, 2 drops of linoleic acid and 100 mL of distilled water were added to the flask with vigorous agitation. About 4.8 mL of this mixture were added to 0.2 mL of each dilution distributed in test tubes. After reading zero time absorbance at 470 nm, the tubes were incubated in a water bath at 50 °C with agitation (100 rpm) for 2h, and the absorbances were measured for the second time at 470 nm (UV–Vis spectrophotometer SPECORD 200, Analytik Jena AG, Jena, Germany) (**Figure 16**). β -carotene bleaching inhibition was calculated through this equation: (absorbance after 2 h of assay/initial absorbance) \times 100 and further converted to EC₅₀ (extract concentration responsible for 50% of β -carotene bleaching inhibition), expressed in mg/mL of extract.



Figure 16. Test tubes used in the β -carotene/linoleate assay.

5.1.4 Thiobarbituric acid reactive substances (TBARS) assay

TBARS is a colorimetric assay in which lipid peroxidation produces malondialdehyde (MDA) as secondary breakdown product, and reacts with the thiobarbituric acid (TBA) to form MDA-TBA complex with the production of a pink pigment (Ndhlala et al., 2010).

The inhibition of lipid peroxidation in porcine (*Sus scrofa*) brain homogenates in the presence of antioxidant is detected by measuring the absorbance of MDA-TBA complex at 532 nm.

Porcine brains were purchased from a local slaughter house, dissected and dissolved in Tris-HCl buffer (20 mmol/L, pH 7.4) in a proportion of 1:2 w/v brain tissue homogenate which was centrifuged at 3000 g for 10 min. Each dilution of the sample solutions (200 µL) was pipetted into test tubes, adding also 100 µL of ascorbic acid (0.1 mmol/L), 100 µL of FeSO₄ (10 mmol/L) and 100 µL of the supernatant of brain tissue homogenate. The tubes were incubated at 37 °C for 1h. Trichloroacetic acid (500 µL, 28% w/v) was added to stop reaction, together with 380 µL thiobarbituric acid (TBA, 2% w/v), and the mixture was then incubated at 80 °C for 20 min. The mixtures were centrifuged at 3500 rpm for 5 min to eliminate the precipitated protein; the absorbances of the supernatant samples were measured at 532 nm (**Figure 17**). The percentage of inhibition was calculated through this equation: inhibition ratio (%) = $[(A-B)/A] \times 100$; (A- absorbance of the control and B- absorbance of the sample solution) and converted to EC₅₀ values (extract concentration responsible for 50% of lipid peroxidation inhibition) expressed in mg/mL of extract.



Figure 17. Test tubes used in the TBARS assay.

5.2 Anti-inflammatory activity

The final extracts were dissolved in water at 8 mg/mL and several dilutions were obtained from the stock solutions 0.005 to 0.4 mg/mL.

5.2.1 Cells treatment

The mouse macrophage-like cell line RAW264.7 was cultured in DMEM medium supplemented with 10% heat-inactivated foetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin and were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. For each experiment, cells were detached with a cell scraper. Under our experiment cell density (5×10^5 cells/mL), the proportion of dead cells was less than 1%, according to Trypan blue dye exclusion tests.

Cells were seeded in 96-well plates at 150.000 cells/well and allowed to attach to the plate overnight. Then, cells were treated with the different concentrations of each of the extracts for 1h. Dexamethasone (50 µM) was used as a positive control for the experiment. The following step was stimulation with LPS (1 µg/mL) for 18h. The effect of all the tested samples in the absence of LPS was also evaluated, in order to observe if they induced changes in NO basal levels. In negative controls, no LPS was added. Both extracts and LPS were dissolved in supplemented DMEM.

5.2.2 Nitric oxide determination

For the determination of nitric oxide, Griess Reagent System kit (Promega) was used, which contains sulfanilamide, N-(1-naphthyl) ethylenediamine hydrochloride (NED) and nitrite solutions. A reference curve of the nitrite (sodium nitrite 100 µM to 1.6 µM; $y=0.0064x+0.1311$, $R^2=0.9981$) was prepared in a 96-well plate. One hundred microliters of the cell culture supernatant was transferred to the plate in duplicate and mixed with sulfanilamide and NED solutions, 5-10 minutes each, at room temperature.

The nitric oxide produced was determined by measuring the absorbance at 540 nm using microplate reader described above (**Figure 18**), and by comparison with the standard calibration curve. The percentage of inhibition of the NO production was calculated, for each sample concentration, by the equation: $[(\text{Substrate concentration} - \text{Basal cells}) / \text{LPS} - \text{Basal cells}] \times 100$.

For an easier comparison of the results, EC₅₀ values were calculated based on 50% of inhibition of NO production (expressed in µg/mL extract).

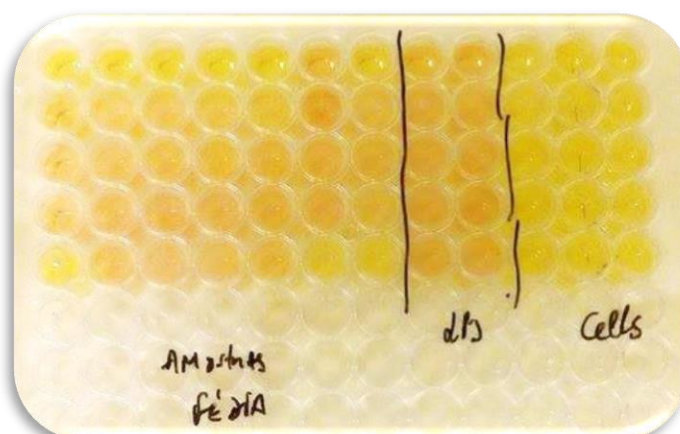


Figure 18. Microplate showing the NO assay with methanolic extracts.

5.3 Cytotoxic activity

The final extracts were dissolved in water at 8 mg/mL and several dilutions were obtained from the stock solutions: 0.125 to 0.4 mg/mL. Ellipticine was used as positive control.

5.3.1 In human tumor cell lines

Four human tumor cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma).

The cell lines were maintained with RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), glutamine (1%, 2 mM) and antibiotic (1%) at 37 °C, in a humidified air incubator containing 5% CO₂ for 24h. The medium was changed every 2 days. The cell concentrations were determined by counting in a hemacytometer chamber under a microscope using trypan blue solution.

For the determination of cell density, sulforhodamine B assay was used (Guimaraes et al., 2013). In brief, the cell lines were cultured at an appropriate density (1×10^4 cells/mL per well in 96-well plates) and then treated with the different extract solution at 37°C in a humidified incubator with 5% CO₂ for 48h. After incubation, cells were fixed with 100 μ L of 10% cold trichloroacetic acid (TCA) and maintained at 4°C for 1h. The plates were then washed with slow-running tap water and dried. Sulforhodamine B solution (0.1% in 1% acetic acid, 100 μ L) was added to each plate well and left for 30 min at room temperature, and then quickly rinsed the plates with 1% acetic acid to remove unbound dye. The protein-bound dye was

solubilized in 200 μ l of 10 mM Tris base solution for the absorbance determination at 540 nm using the microplate reader mentioned above (**Figure 19**).

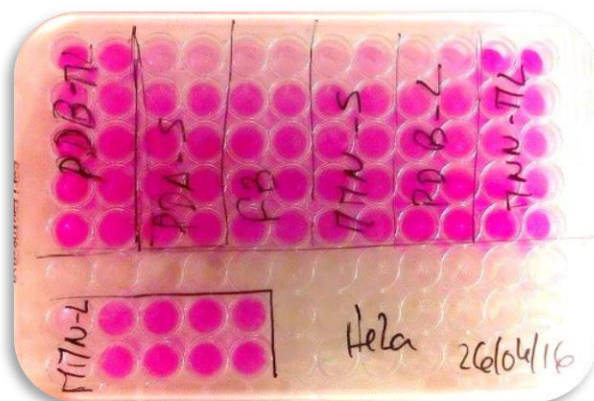


Figure 19. Microplate used in the evaluation of the methanolic extracts cytotoxicity.

The extract concentration that inhibited 50% of the net cell growth (GI_{50}) was calculated from the graph of sample concentration against percentage of growth inhibition and expressed in μ g/mL extract.

5.3.2 In non-tumor cells

The effect of the extracts on the growth of porcine liver primary cells (PLP2), established by the group, was evaluated by the sulforhodamine B colorimetric assay with some modifications as described by Abreu et al., (2011). Briefly, the liver tissues were rinsed in Hank's balanced salt solution containing 100 U/mL penicillin and 100 μ g/mL streptomycin and divided into 1×1 mm³ explants. Some of these explants were placed in 25 cm³ tissue flasks in DMEM supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin and incubated at 37 °C with a humidified atmosphere containing 5% CO₂. The medium was changed every 2 days. Cultivation of the cells was continued with direct monitoring every 2-3 days using a phase contrast microscope. Before confluence, cells were sub-cultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin. Cells were treated for 48 h with the different diluted sample solutions and the SRB assay was performed. The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth) in μ g/mL extract.

6. Statistical

For each culture component, fruiting body and fungal species, three independent samples were analysed. Data were expressed as mean \pm standard deviation. All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., USA).

The fulfilment of the one-way ANOVA requirements, specifically the normal distribution of the residuals (data not shown) and the homogeneity of variance, was tested by means of the Shapiro Wilk's and the Levene's tests, respectively. All dependent variables were compared using Tukey's honestly significant difference (HSD) or Tamhane's T2 multiple comparison tests, when homoscedasticity was verified or not, respectively.

CHAPTER 4. RESULTS AND DISCUSSION

1. Mycelia growth

Edible mushrooms, in general, are esteemed primarily for their nutritional value and bioactive properties mostly provided by different active substances, such as polysaccharides, lipids, peptides, sterols, or fiber (Ren et al., 2016). The great majority of the studies reporting the previous features are conducted on the fruiting body, but the mycelia, as well as the culture media utilized in different stages of mushroom production, might also represent a good source of valuable compounds.

Besides the differences in bioactive compounds and corresponding activities, each strain of mushroom produces a special type of mycelium and this range of characteristics varies in form, color and growth rate. *P. eryngii* presented depigmented and cottony mycelia, whereas *S. bellinii* had pigmented and rhizomorphic mycelia (**Figure 20**).

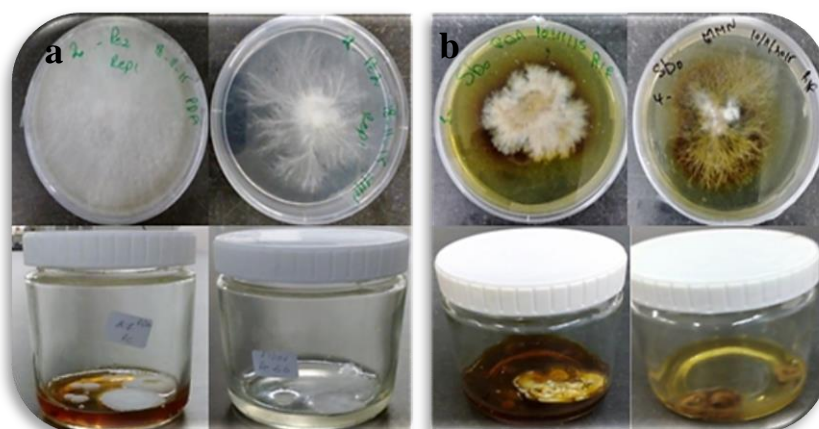


Figure 20. *Pleurotus eryngii* (a) and *Suillus bellinii* (b) mycelia cultivated *in vitro*, in both liquid (PDB and iMMN) and solid medium (PDA and iMMN).

The growth rate and yielded biomass of mycelia are of paramount importance, since these parameters might define the industrial interest of each species. Accordingly, both indicators are presented in **Figures 21, 22 and 23**, to allow a proper assessment of their true applicability. *P. eryngii* and *S. bellinii* mycelia started to grow after 3 days of culture. For *P. eryngii*, the mycelium grown in PDA media showed the fastest radial growth and for *S. bellinii*, the mycelium grown in iMMN solid media showed the fastest radial growth. These results are in agreement with the already known better growth of mycorrhizal fungi in MMN medium when compared with PDA medium (Marx, 1969). The opposite is true for nonmycorrhizal species like *P.eryngii*.

Overall, *P. eryngii* presented a higher growth rate, despite the similarities in the produced biomass (except for the mycelia grown in PDB). This result is also in agreement with the known better *in vitro* growth of nonmycorrhizal fungi when compared to mycorrhizal. Symbiotic fungi are often very dependent on the host for survival and growth and require richer growth media. This was the reason for the development of MMN medium by Marx, (1969). Similarities in biomass production make the applicability of *S. bellinii* particularly interesting in case of positive bioactivity results for these mycelia.

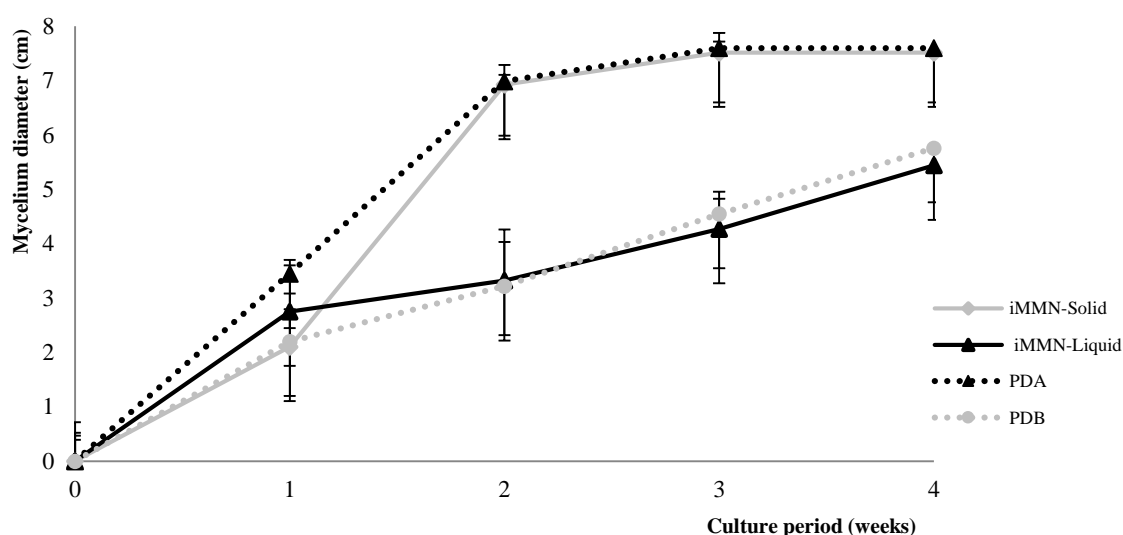


Figure 21. Growth in diameter of the mycelia of *P. eryngii* cultivated in different culture media throughout time.

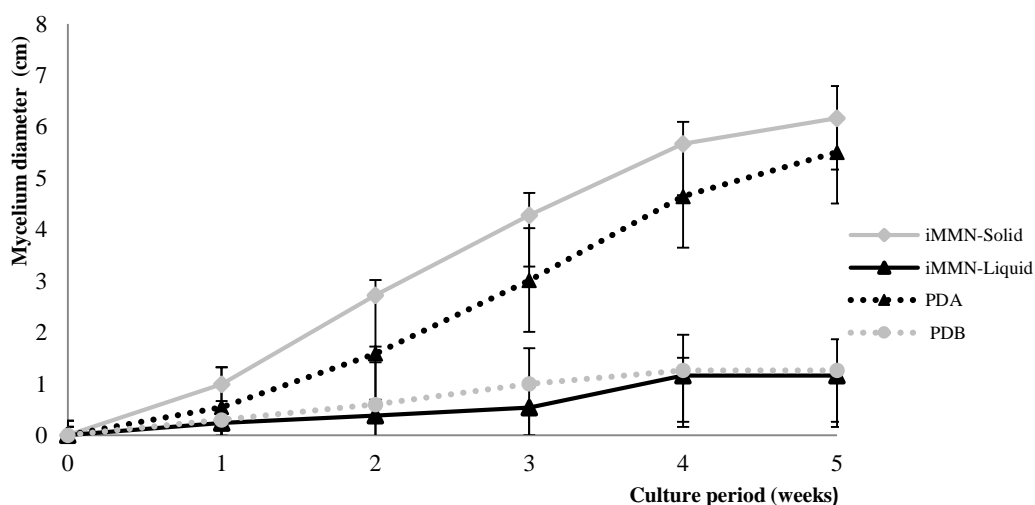


Figure 22. Growth in diameter of the mycelia of *S. bellinii* cultivated in different culture media throughout time.

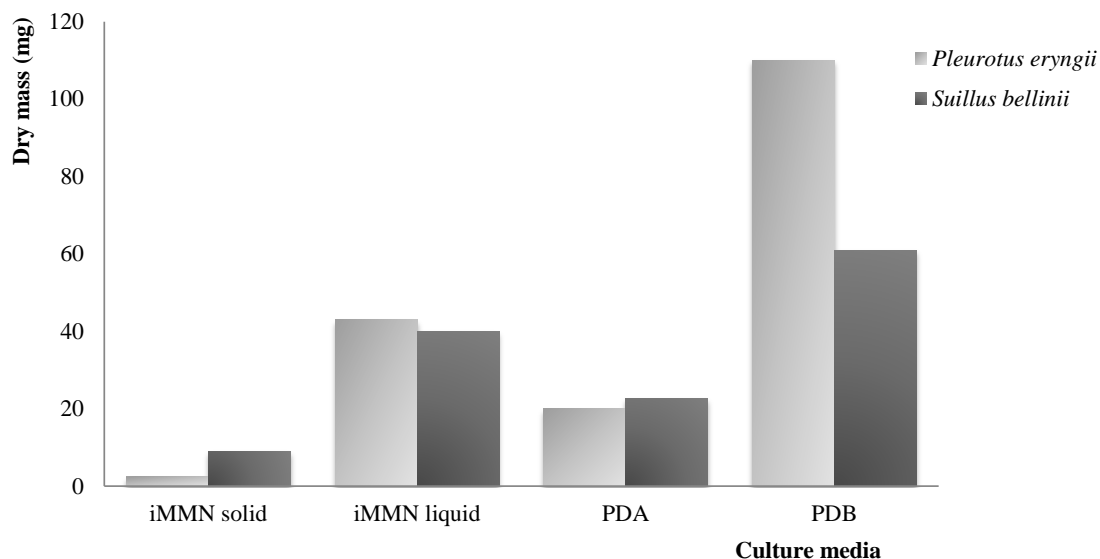


Figure 23. Average biomass of *P. eryngii* and *S. bellinii* mycelia (mg/Petri dish or Flask).

In the following sections, the mycelia and culture media are compared by evaluating different bioactive compounds and bioactivity indicators. In all tables, results corresponding to wild samples of each studied species are also presented as reference values.

2. Chemical characterization of the extracts

The phenolic acids profile and ergosterol contents are presented in **Table 6**. In general, the fruiting body presented higher contents in phenolic acids, but it was very interesting to find that the mycelium of *S. bellinii* (independently of the culture conditions) gave higher contents (8.9-12.4 mg/g extract) in ergosterol than the corresponding wild samples (6.5 mg/g extract). There are limited data on the sterols content of *S. bellinii*, but it was reported as having 12 mg/100 g fw ergosterol (Kalogeropoulos et al., 2013), which is higher than the values reported in the present work.

P. eryngii gave lower contents in ergosterol, either considering the mycelia (0.10-1.0 mg/g extract), as well as the fruiting body (4.1 mg/g extract), when compared to *S. bellinii*. The ergosterol content in *P. eryngii* fruiting body was previously reported as 20 mg/100 g dw (Barreira et al., 2014), but that higher value was measured in commercial samples, which might justify the difference in comparison to the result reported here.

Among the phenolic acids, *p*-hydroxybenzoic acid was the major compound in both mushrooms, reaching quantities nearly sevenfold higher in *S. bellinii* fruiting body (1821 µg/g extract). Nevertheless, the main phenolic acid in *S. bellinii* was previously reported as being

p-hydroxyphenylacetic acid (45 µg/100 g fresh weight), among *p*-hydroxybenzoic acid derivatives, and *o*-coumaric acid (15 µg/100 g fresh weight), in what concerns hydroxycinnamic acids. In the same study, the concentration of *p*-hydroxybenzoic was 7 µg/100 g fresh weight (Kalogeropoulos et al., 2013), which is in the range of the concentrations detected herein, considering typical moisture contents (≈90%) and extraction yields (≈5%) for this particular mushroom species.

Concerning *P. eryngii*, the phenolic acids profile is in agreement with previous works conducted with samples from the same geographic area, but different harvesting years (Ferreira et al., 2009; Heleno et al., 2015; Reis et al., 2014). Even so, in a similar study, syringic acid and vanillic acid were reported in quantities similar to *p*-hydroxybenzoic acid (Lin et al., 2014).

Table 6. Ergosterol content (mg/g extract) and phenolic acids composition (µg/g extract) in the mycelia and culture media of *P. eryngii* and *S. bellinii*. The values corresponding to the fruiting body of both mushrooms (wild samples) are also presented. Values are given as mean±standard deviation.

		Ergosterol	Protocatechuic acid	<i>p</i> -Hydroxybenzoic acid	<i>p</i> -Coumaric acid	Cinnamic acid
<i>Pleurotus eryngii</i>						
Fruiting body (wild)		4.1±0.3	nd	273±10	42±4	61±4
iMMN liquid	Mycelium	0.6±0.1 ^b	nd	186±8 ^a	nd	34±3 ^b
	Culture medium	nd	nd	nd	nd	nd
PDB	Mycelium	0.10±0.02 ^c	nd	122±6 ^c	nd	49±4 ^a
	Culture medium	nd	nd	nd	nd	nd
iMMN solid Mycelium		0.9±0.1 ^a	nd	149±9 ^b	nd	15±2 ^c
PDA	Mycelium	1.0±0.1 ^a	nd	178±8 ^a	nd	15±2 ^c
<i>p</i> -values (n = 54)	Homoscedasticity ¹	<0.001	-	<0.001	-	<0.001
	1-way ANOVA ²	<0.001	-	<0.001	-	<0.001
<i>Suillus bellinii</i>						
Fruiting body (wild)		6.5±0.4	403±16	1821±97	nd	39±4
iMMN liquid	Mycelium	8.9±0.5 ^b	nd	213±7 ^c	nd	130±1 ^a
	Culture medium	nd	nd	nd	nd	nd
PDB	Mycelium	12.4±0.5 ^a	nd	204±11 ^c	nd	111±4 ^b
	Culture medium	nd	nd	nd	nd	nd
iMMN solid Mycelium		9.0±0.4 ^b	nd	394±15 ^a	nd	25±2 ^d
PDA	Mycelium	3.5±0.3 ^c	nd	372±16 ^b	nd	36±3 ^c
<i>p</i> -value (n = 54)	Homoscedasticity ¹	<0.001	-	<0.001	-	<0.001
	1-way ANOVA ²	<0.001	-	<0.001	-	<0.001

nd- not detected. ¹Homoscedasticity among culture components was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. ² $p<0.05$ indicates that the mean value of at least one component differs from the others (in this case, multiple comparison tests were performed). For each culture component, means within a column with different letters differ significantly ($p<0.05$).

3. Antioxidant activity

The results obtained for each of the performed antioxidant activity assays, given as EC₅₀ values, are shown in **Table 7**. In all evaluated cases, the highest activity was obtained on the TBARS formation inhibition assay (*P. eryngii*: 0.11 mg/mL extract; *S. bellinii*: 0.011 mg/mL extract), followed by β -carotene bleaching inhibition (*P. eryngii*: 0.45 mg/mL extract; *S. bellinii*: 0.12 mg/mL extract), reducing power (*P. eryngii*: 0.98 mg/mL extract; *S. bellinii*: 0.16 mg/mL extract) and scavenging effects on DPPH radicals (*P. eryngii*: 12.8 mg/mL extract; *S. bellinii*: 0.61 mg/mL extract). In line with its higher quantities of phenolic acids, the fruiting bodies of *S. bellinii* showed higher antioxidant activity than *P. eryngii*, sometimes 10-fold or 20-fold higher, such as verified in the TBARS formation inhibition and DPPH scavenging activity, respectively. In general, the values obtained for *P. eryngii* represent higher antioxidant activity (except for the DPPH scavenging activity assay) than that reported previously (Reis et al., 2012; Lin et al., 2014).

In this context, phenolic acids are described as being important compounds with antioxidant activity in mushrooms due to the presence of OH groups in their chemical structures that are known for their ability to scavenge free radicals (Heleno et al., 2012a).

Regarding *S. bellinii*, an activity of 35% in DPPH scavenging activity, at 0.15 mg/mL was previously reported (Ribeiro et al., 2006), which is slightly better than the 50% activity obtained in this study for the 0.61 mg/mL concentration. In a similar study, performed with Greek samples of *S. bellinii*, this mushroom also showed high radical scavenging activity and reducing power, but the results are not directly comparable, because they were given in trolox equivalents (Kalogeropoulou et al., 2013).

Considering the main purpose of this work, it was very interesting to discover that the antioxidant activity measured in the mycelia and in the culture media was very close (in some cases better) to that verified in the fruiting bodies, emphasizing the high potential of these fungal culture components. In the case of the culture media, these results have an increased interest, since those components are usually considered as by-products of mushroom cultivation. Furthermore, the differences among the same culture media, after having been used to grow each of the mushrooms, indicate that the measured antioxidant activity is in fact due to the mycelium, and not to the culture media components. Furthermore, some

compounds responsible for the antioxidant activity are not released to the culture medium; they are kept in the mycelium. Without exception, the mycelia and the culture media of *S. bellinii* gave better results than the same components of *P. eryngii*.

Table 7. Antioxidant activity (EC₅₀ values, mg/mL extract) of the mycelia and culture media of *P. eryngii* and *S. bellinii*. The values corresponding to the fruiting body of both mushrooms (wild samples) are also presented. Values are given as mean±standard deviation

		DPPH scavenging activity	Reducing power	β-carotene bleaching inhibition	TBARS formation inhibition
<i>Pleurotus eryngii</i>					
Fruiting body (wild)		12.8±0.2	0.98±0.01	0.45±0.02	0.11±0.01
iMMN liquid	Mycelium	24.9±0.3 ^b	1.35±0.02 ^a	0.44±0.03 ^c	0.17±0.01 ^b
	Culture medium	28.2±0.5 ^a	1.24±0.05 ^b	0.57±0.01 ^a	0.20±0.02 ^a
PDB	Mycelium	19.7±0.5 ^d	1.10±0.01 ^c	0.40±0.05 ^c	0.15±0.01 ^c
	Culture medium	22.0±0.5 ^c	0.95±0.02 ^e	0.52±0.02 ^b	0.17±0.01 ^b
iMMN solid	Mycelium	17.1±0.5 ^e	1.08±0.01 ^c	0.23±0.01 ^d	0.13±0.01 ^d
PDA	Mycelium	16.1±0.5 ^f	1.03±0.01 ^d	0.18±0.02 ^e	0.11±0.01 ^e
<i>p</i> -value (n = 54)	Homoscedasticity ¹	0.015	<0.001	<0.001	<0.001
	1-way ANOVA ²	<0.001	<0.001	<0.001	<0.001
<i>Suillus bellinii</i>					
Fruiting body (wild)		0.61±0.01	0.16±0.01	0.12±0.01	0.011±0.001
iMMN liquid	Mycelium	1.10±0.01 ^b	0.84±0.02 ^b	0.21±0.03 ^b	0.14±0.01 ^a
	Culture medium	1.18±0.02 ^a	0.93±0.02 ^a	0.29±0.01 ^a	0.15±0.01 ^a
PDB	Mycelium	0.83±0.02 ^d	0.24±0.01 ^d	0.17±0.01 ^d	0.010±0.001 ^c
	Culture medium	0.95±0.03 ^c	0.29±0.02 ^c	0.20±0.01 ^c	0.014±0.002 ^{bc}
iMMN solid	Mycelium	0.62±0.03 ^e	0.17±0.01 ^e	0.14±0.02 ^e	0.016±0.001 ^b
PDA	Mycelium	0.59±0.02 ^f	0.16±0.01 ^e	0.14±0.01 ^e	0.011±0.001 ^c
<i>p</i> -value (n = 54)	Homoscedasticity ¹	0.003	<0.001	<0.001	<0.001
	1-way ANOVA ²	<0.001	<0.001	<0.001	<0.001

¹Homoscedasticity among culture components was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. ² $p<0.05$ indicates that the mean value of at least one component differs from the others (in this case, multiple comparison tests were performed). For each culture component, means within a column with different letters differ significantly ($p<0.05$). Trolox EC₅₀ values: 41.68±0.28 µg/ mL (DPPH scavenging activity), 41.43±1.27 µg/ mL (reducing power), 18.21±1.12 µg/ mL (β-carotene bleaching inhibition) and 22.84±0.74 µg/ mL (TBARS inhibition).

In coherence with the observed for the fruiting bodies, the results obtained with *P. eryngii* mycelia were better than the reported previously, except for DPPH scavenging activity (Reis et al., 2012).

In general, the detected antioxidant activity represents an important added-value, despite the weaker performance of the mycelium and culture medium extracts when compared with the DPPH scavenging activity (EC_{50} values ranging from 0.8-3 mg/mL) and reducing power ($EC_{50} = 0.5$ mg/mL) obtained in polysaccharide fractions of *P. eryngii* (Li & Shah, 2014; Ma et al., 2016; Zhang et al., 2016). However, it should be reminded that the results presented herein were obtained with raw extracts, not with purified fractions.

Due to the lack of studies with the mycelia of *S. bellinii*, no comparisons could be performed.

4. Anti-inflammatory activity

Macrophages, the main components of the innate immune system, have essential regulation functions in several immunopathological conditions during the inflammatory process (Yoon et al., 2009; Yuan et al., 2006). However, the overproduction of inflammatory mediators in the course of uncontrolled inflammation processes might cause adverse consequences in the pathogenesis of many inflammatory diseases such as cancer, diabetes and cardiovascular disease (Yuan et al., 2006). Endotoxin lipopolysaccharide (LPS) is able to induce the production of mediators like nitric oxide (NO), pro-inflammatory cytokines (besides inhibiting anti-inflammatory cytokines) and tumor necrosis factor in macrophages. Thereby, macrophages stimulated by LPS have been widely used for anti-inflammatory activities evaluation *in vitro* (García-Lafuente et al., 2009). In addition, due to reproducible response of RAW264.7 macrophages to LPS, this cell line has been widely used for inflammatory research (Huang & Ho, 2010).

Accordingly, the anti-inflammatory activity was evaluated using a LPS-stimulated RAW264.7 cell line (**Table 8**). The highest activity was measured in the methanolic extracts prepared from the fruiting bodies of *S. bellinii* ($EC_{50} = 90$ µg/mL extract). The extracts of *P. eryngii* fruiting bodies were also able to suppress NO production, but in less extent ($EC_{50} = 223$ µg/mL extract). However, these results were better than those obtained in previous assays (Taofiq et al., 2015).

Regarding the evaluated culture components, some interesting results were obtained, especially for the mycelia of *P. eryngii* grown in solid media, which showed higher anti-inflammatory activity ($EC_{50} = 184$ -189 µg/mL extract) than the corresponding fruiting bodies, highlighting their possible use in anti-inflammatory applications.

Moro et al., (2012) found that the phenolic compounds of the fruiting bodies might contribute to their anti-inflammatory activities, inducing inhibition of NO production and iNOS expression in LPS-activated RAW264.7 cells.

Positive interactions could be observed between phenolic acids (in particular, *p*-hydroxybenzoic acid) and anti-inflammatory effects of *P. eryngii* fruiting body and mycelia. It was observed that the extract with the highest anti-inflammatory activity showed the highest levels of phenolic acids and ergosterol. However, the anti-inflammatory activity of the mycelia of *S. bellinii* was not maintained in neither of the culture components, indicating that the anti-inflammatory effects of the extracts appeared to be related with other components besides ergosterol and phenolic acids.

5. Cytotoxic activity

The results for the anti-proliferative activity assayed in four human tumor cell lines (MCF-7, NCI-H460, HeLa and HepG2) and a porcine liver primary cell line (PLP2) are shown in **Table 8**.

The extracts prepared from the fruiting bodies of wild *S. bellinii* samples showed higher activity against MCF7 ($GI_{50} = 70 \mu\text{g/mL}$ extract), NCI-H460 ($GI_{50} = 65 \mu\text{g/mL}$ extract) and HepG2 ($GI_{50} = 68 \mu\text{g/mL}$ extract). However, the same extracts could not inhibit the HeLa cell line (up to the maximum assayed concentration: $400 \mu\text{g/mL}$ extract). On the other hand, the extracts from the fruiting bodies of wild *P. eryngii* samples had a similar behavior in all cell lines (GI_{50} values varying from $224 \mu\text{g/mL}$ extract in HepG2 to $246 \mu\text{g/mL}$ extract in MCF7). The anti-proliferative activity of *P. eryngii* extracts (specifically, its polysaccharides fraction) was previously reported in HepG2, where it induced apoptosis, cell cycle arrest at the S-phase and intracellular production of reactive oxygen species (Yang et al., 2013). Besides its *in vitro* anti-tumoral activity, *P. eryngii* was previously reported as being active against mice renal cancer *in vivo* (Yang et al., 2013). Despite their generally lower activity against tumor cell lines, the extracts from *P. eryngii* did not exhibit a toxic effect on the primary cell line, contrarily with the observed for *S. bellinii* fruiting bodies.

Interestingly, none of the assayed culture components inhibit the growth of PLP2 cell line, which constitutes a good indicator of the lack of toxicity of the mycelia and culture media of both mushroom species in non-tumor cell lines.

Table 8. Anti-proliferative (GI₅₀ values, µg/mL extract) and anti-inflammatory activity (EC₅₀ values, µg/mL extract) of the mycelia and culture media of *P. eryngii* and *S. bellinii*. The values corresponding to the fruiting body of both mushrooms (wild samples) are also presented. Values are given as mean±standard deviation.

		MCF-7	NCI-H460	HeLa	HepG2	PLP2	RAW264.7
<i>Pleurotus eryngii</i>							
Fruiting body (wild)		246±6	237±10	243±10	224±12	>400	223±9
iMMN	Mycelium	242±7 ^c	275±12 ^{cd}	265±4 ^d	280±3 ^c	>400	>400 ^a
liquid	Culture medium	>400 ^a	>400 ^a	>400 ^a	>400 ^a	>400	>400 ^a
PDB	Mycelium	270±15 ^b	268±5 ^d	280±1 ^c	224±11 ^d	>400	308±10 ^b
	Culture medium	>400 ^a	>400 ^a	>400 ^a	>400 ^a	>400	>400 ^a
iMMN	mycelium	169±12 ^d	363±13 ^b	302±9 ^b	314±2 ^b	>400	189±7 ^c
solid							
PDA	mycelium	173±8 ^d	285±10 ^c	267±20 ^d	140±6 ^e	>400	184±6 ^c
<i>p</i> -value	Homoscedasticity	<0.001	<0.001	<0.001	<0.001	-	<0.001
(n = 54)	1-way ANOVA	<0.001	<0.001	<0.001	<0.001	-	<0.001
<i>Suillus bellinii</i>							
Fruiting body (wild)		70±2	65±3	>400	68±3	215±38	90±2
iMMN	Mycelium	>400 ^a	>400 ^a	>400	>400 ^a	>400	>400
liquid	Culture medium	>400 ^a	>400 ^a	>400	>400 ^a	>400	>400
PDB	Mycelium	300±7 ^b	279±2 ^b	>400	226±5 ^b	>400	>400
	Culture medium	>400 ^a	>400 ^a	>400	>400 ^a	>400	>400
iMMN	Mycelium	>400 ^a	>400 ^a	>400	>400 ^a	>400	>400
solid							
PDA	Mycelium	289±11 ^c	257±2 ^c	>400	206±11 ^c	>400	>400
<i>p</i> -value	Homoscedasticity	<0.001	<0.001	-	<0.001	-	-
(n = 54)	1-way ANOVA	<0.001	<0.001	-	<0.001	-	-

¹Homoscedasticity among culture components was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. ² $p<0.05$ indicates that the mean value of at least one component differs from the others (in this case, multiple comparison tests were performed). For each culture component, means within a column with different letters differ significantly ($p<0.05$). Ellipticine GI₅₀ value: 0.91±0.04 µg/mL (MCF-7), 1.03±0.09 µg/mL (NCI-H460), 1.91±0.06 µg/mL (HeLa), 1.14±0.21 µg/mL (HepG2) and 3.22±0.67 µg/mL (PLP2). Dexamethaxone EC₅₀ value: 15.70±1.1 µg/mL.

Concerning the tumor cell lines, the mycelia of *P. eryngii* showed similar cytotoxicity to the fruiting bodies. In fact, the measured activity was often higher than the observed for the fruiting bodies. In some cases, such as the mycelium grown in PDA medium, the GI₅₀ values are comparable to those obtained with purified polysaccharide fractions (Ma et al., 2014, 2016; Ren et al., 2016), which represents a very interesting result.

On the other hand, the mycelia of *S. bellinii* showed significantly lower anti-proliferative activity, when compared to the fruiting bodies.

The culture media did not show any anti-proliferative activity (up to the maximum assayed concentrations) in both mushroom species.

P. eryngii samples that showed the highest cytotoxic activity have the highest content in phenolic acids and ergosterol.

CHAPTER 5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This study was initially designed to evaluate the mycelia of *Pleurotus eryngii* and *Suillus bellinii*, as well as their culture media, as potential alternative sources of bioactive compounds or as ingredients to be used in applications with antioxidant, anti-inflammatory or cytotoxic activities.

According to the obtained results, we have demonstrated that the growth rate of mycelia depends on the culture media. Potato dextrose agar (PDA) media was the best one for *P. eryngii*, while incomplete Melin-Norkans medium (iMMN) solid media proved to be the best one for *S. bellinii*. Growth rate is not correlated with the biomass of mycelium; for instance, the produced biomass is similar in all culture media (except for the mycelia grown in PDB).

For the chemical analysis, methanolic extracts showed several phenolic acids (*p*-hydroxybenzoic acid, *p*-coumaric acid and protocatechuic acid), a related compound (cinnamic acid) and ergosterol, which have been suggested to play an important role in antioxidant, anti-inflammatory and anti-tumor activities.

In general, *S. bellinii* mycelia showed higher contents of ergosterol (8.9-12.4 mg/g extract) and phenolic acids, mostly *p*-hydroxybenzoic acid (204-394 µg/g extract), which was also more abundant in the fruiting body of this species (1821 µg/g extract). Likewise, the antioxidant activity was also higher among the *S. bellinii* components with the highest activity being obtained on the TBARS formation inhibition assay ($EC_{50} = 0.011$ mg/mL extract). However, these extracts did not show anti-inflammatory activity up to the maximum assayed concentrations, contrarily to the observed for the mycelia of *P. eryngii*. Furthermore, the latter component showed a cytotoxicity similar (and often superior) to its fruiting bodies, in opposition to *S. bellinii*, whose mycelia showed a significant loss of anti-proliferative activity comparing to its fruiting bodies that revealed higher activity against MCF7 ($GI_{50} = 70$ µg/mL extract), NCI-H460 ($GI_{50} = 65$ µg/mL extract) and HepG2 ($GI_{50} = 68$ µg/mL extract). Therefore, it is difficult to assess which compounds are relevant for anti-inflammatory or anti-tumor activities of the studied wild sample.

In general, each culture component showed differentiated activity, which should be considered together with the growth rate and biomass yielded for each mushroom.

In vitro culture could be explored to obtain bioactive compounds from fruiting body used as unmodified natural products or in nutraceutical formulations with antioxidant, anti-inflammatory and anti-tumor activities, as a possible food supplement or as an ingredient for industrial applications maintaining and promoting health and life quality.

In this context, genomic, proteomic and metabolomic tools should be used to assist further *in vitro* cultivation in order to conclude about the most promising bioactive molecules to develop novel pharmaceutical products with a positive global impact on human healthcare but maintaining environmental preservation.

The safety criteria of these compounds has been revealed by the absence of hepatotoxicity in porcine liver primary cells (PLP2), but *in vivo* models should be carried out in future steps.

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