Development of nutraceutical formulations based on mycelium of *Pleurotus ostreatus* and *Agaricus bisporus*

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LIST OF ABREVIATIONS

5-LOX: 5-Lipooxygenase

ANOVA: One vary analysis of variance

B2: Riboflavin

B3: Niacin

B5: Pantothenic acid

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoluene

COX-2: Cyclooxygenase-2

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

DNA: Deoxyribonucleic acid

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

Dw: Dry weight

EC$_{50}$: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in reducing power assay

ECACC: European collection of animal cell culture

FAO: Food and Agriculture Organization

FBS: Fetal bovine serum

Fw: Fresh weight

GAE: Gallic acid equivalents

GI$_{50}$: Extract concentration that inhibited 50% of the net cell growth

HBSS: Hank's balanced salt solution

HBV: Hepatitis B virus

HCV: Hepatitis C virus

HeLa: Henrietta Lacks human cervical carcinoma cell line

HepG2: Hepatocellular carcinoma cell line

HIV: Human immunodeficiency virus

HPLC: High performance liquid chromatography

HPLC-DAD: Diode array detector

HPLC-UV: Ultraviolet detector

HPV: Human papillomavirus

HSD: Tukey's honestly significant difference

IAEA: International Atomic Energy Agency

IC$_{50}$: Extract concentration corresponding to 50% of cytotoxic activity

ICAM-1: Intercellular adhesion molecule-1

IFN-δ: Interferon gamma

IL-1 β: Interleukin 1β

IL-6: Interleukin 6

IL-8: Interleukin 8

IL-12: Interleukin 12

iMMN: Incomplete melin-norkans medium

iNOS: Inducible nitric oxide synthase

LPS: Lipopolysaccharide

MA: Massachusetts

MCF-7: Breast adenocarcinoma cell line

MD: Maryland

MDA: Malondialdehyde

MMN: Melin–norkans medium

NCI-H460: Human lung cancinoma cell line

NF-KB: Nuclear factor kappa B

NED: N-(1-napthyl) ethylenediamine hydrochloride

NK-cell: Natural killer cell

NSAIDs: Nonsteroidal anti-inflammatory drugs

PDA: Potato dextrose agar medium

PDB: Potato dextrose broth

PLP2: Non-tumor primary culture of porcine liver cells

PG: Propyl gallate

PGE2: Prostaglandin E2

RAW 264.7: Mouse leukaemic monocyte macrophage cell line

RNA: Ribonucleic acid

ROS: Oxygen reactive species

RSA: Radical scavenging activity

SRB: Sulphorodamine B

TBARS: Thiobarbituric acid reactive substances

TBA: Thiobarbituric acid

TBHQ: Tert-butylhydroquinone

TCA: Trichloroacetic acid

TNF-α: Tumor necrosis factor alpha

µl: Microliter

USA: United States of America

UK: United Kingdom

UV: Ultraviolet

UFLC: Ultra-fast liquid chromatography

VT: Vermont

v/v: Volume/Volume

w/v: Weight/Volume

WHO: World Health Organization

ABSTRACT

The importance of improving people's quality of life has aroused the interest of the food industry to develop new products with functional characteristics that may be associated with possible beneficial effects on human health, either preventive or therapeutic aspects. Functional foods and nutraceuticals present in its composition bioactive substances that provide medical benefits and, therefore, have aroused the interest of many consumers around the world. Mushrooms are a source of proteins, vitamins, minerals, and especially bioactive compounds. These compounds, including ergosterol, phenolic compounds, tocopherols, ascorbic acid and carotenoids, have been associated with antioxidant, anti-inflammatory, antimicrobial and cytotoxic properties, among others attributed to mushrooms and associated to its promoting health effects. Following this approach, the present work aimed to develope nutraceutical formulations based on mycelium of *Agaricus bisporus* L. and *Pleurotus ostreatus* (Jacq. Ex Fr.) P. Kumm. The present study highlights the potential of *in vitro* culture as a tool to improve production of bioactive compounds by two different mushroom species. Accordingly, *A. bisporus* and *P. ostreatus* were studied for their composition in phenolic acids and sterols, antioxidant activity (DPPH radicals scavenging, reducing power, β-carotene bleaching inhibition and TBARS formation inhibition), anti-inflammatory effect (by down-regulating LPS-stimulated NO in RAW264.7 cells) and cytotoxic activity (using MCF-7, NCI-H460, HeLa, HepG2 and PLP2 cell lines). Therefore, the mycelia of *A. bisporus* and *P. ostreatus* was cultured in different solid and liquid media, and further submitted to solid-liquid extraction processes; these assayed species showed differences in the growth rate and yielded biomass. Overall, *P. ostreatus* mycelia showed higher contents of ergosterol and phenolic compounds (but the mycelia of *A. bisporus* produced in PDA presented a higher amount of *p*-hydroxybenzoic acid) and stronger antioxidant activity than the corresponding fruting body. On the other hand, *P. ostreatus* and *A. bisporus* did not show anti-inflammatory activity. However, *P. ostreatus* showed cytotoxicity in human tumor cell lines in opposition to *A. bisporus*, that didn't present cytotoxic activity. In conclusion, the results show that these mushrooms are a good source of compounds with antioxidant and cytotoxic capacity, with variations among species.

Keywords: Nutraceutical formulations; Mycelium; Phenolic compounds; Ergosterol; Bioactivity.

RESUMO

A importância de melhorar a qualidade de vida das pessoas tem despertado o interesse da indústria alimentar pelo desenvolvimento de novos produtos com características funcionais que possam estar associados a possíveis efeitos benéficos na saúde humana, quer a nível preventivo, quer terapêutico. Os alimentos funcionais e nutracêuticos apresentam na sua composição substâncias bioativas com efeitos benéficos e, portanto, tornam-se apelativos para muitos consumidores em todo o mundo. Os cogumelos são uma fonte de proteínas, vitaminas, minerais e, muitos outros compostos bioativos. Estes compostos, incluindo ergosterol, compostos fenólicos, tocoferóis, ácido ascórbico e carotenóides, têm sido associados a propriedades antioxidantes, anti-inflamatórias, antimicrobianas e citotóxicas, entre outras, relacionadas com os seus efeitos na promoção da saúde. Assim, o presente trabalho teve por objetivo desenvolver formulações nutracêuticas baseadas em micélio de *Agaricus bisporus* e *Pleurotus ostreatus.* O estudo destaca ainda o potencial da cultura *in vitro* como ferramenta para melhorar a produção de compostos bioativos em duas espécies de cogumelos diferentes. Por conseguinte, *A. bisporus* e *P. ostreatus* foram estudados quanto à sua composição em ácidos fenólicos e esteróis, atividade antioxidante (captação de radicais livres DPPH, poder redutor, inibição da descoloração do *β*-caroteno e inibição da formação de TBARS), efeito anti-inflamatório (inibição da produção de NO em células RAW264.7 estimuladas por LPS) e actividade citotóxica (usando Linhas celulares MCF-7, NCI-H460, HeLa, HepG2 e PLP2). Os micélios de *A. bisporus* e *P. ostreatus* foram cultivados em diferentes meios de cultura, sólidos e líquidos, e posteriormente submetidos a processos de extracção sólido-líquido; estas espécies analisadas apresentaram diferenças na taxa de crescimento e rendimento de biomassa. Em geral, o micélio de *P. ostreatus* apresentou teores mais elevados de compostos fenólicos e ergosterol (mas o micélio de *A. bisporus* produzido em PDA apresentou uma maior quantidade de ácido *p*-hidroxibenzóico) e maior atividade antioxidante do que o corpo de frutificação correspondente. Por outro lado, *A. bisporus* e *P. ostreatus* não mostraram nenhuma atividade anti-inflamatória. No entanto, *P. ostreatus* mostrou citotoxicidade em linhas celulares tumorais humanas, ao contrário de *A. bisporus* que não apresentou atividade citotóxica. Em conclusão, os resultados mostram que estes cogumelos são uma boa fonte de compostos com capacidade antioxidante e citotóxica, com variações entre espécies.

Palavras-chave: Formulações nutracêuticas; Micélio; Compostos fenólicos; Ergosterol; Bioatividade.

CHAPTER 1.

1. INTRODUTION

In recent decades the demands of consumers in terms of food production have changed considerably. Consumers increasingly believe that food directly contribute to their health and well-being. Today's foods are not only intended to satisfy hunger and provide the necessary nutrients, but also to prevent disease and improve physical and mental well-being of consumers. In this context, functional foods play a very important role. The growing demand for such foods can be explained by the rapid advances in science and technology, increasing health care costs, changes in effective food laws tam labels and product claims, the steady increase in life tenancy, the desire of older people to improving quality of life in their late years and the increased interest in maintaining good health through diet (Siro *et al.,* 2008).

The subject "nutraceuticals" and "functional foods" is experiencing a broad discussion in the scientific community, and with the recent improvements and biotechnological discoveries of recent decades, it becomes a recent research hot topic.

According to DeFelice (1995) and subsequent references, "a nutraceutical is any substance that is a food or a part of food and provides medical or health benefits, including the prevention and treatment of disease" (DeFelice, 1995; Brower, 1998 and Rishi, 2006). Foods, known as "functional foods," are thought to provide benefits beyond basic nutrition and may play a role in reducing or minimizing the risk of certain diseases and other health conditions (Cencic & Chingwaru, 2010). Nutraceuticals and functional characteristics of many traditional foods are being discovered and studied, while new food products are being developed to include beneficial components. By knowing which foods can provide specific health benefits, we can make food and beverage choices that allow us to take greater control of our health.

Mushrooms belong to the kingdom Fungi, a group very distinct from plants, animals and bacteria. Fungi depend on other organisms for food, absorbing nutrients from the organic material in which they reside (Adamovic *et al.,* 1998).

Mushrooms have shown many biotechnological potential and have attracted the attention

of the pharmaceutical and food industries due to its nutritional value and the presence of numerous bioactive substances (Kalac, 2009). These molecules are responsible for the use of mushrooms as important partners in the complementary treatment of many diseases such as cancer, hepatitis, human papillomavirus (HPV) and human immunodeficiency virus (HIV). In addition to these therapeutic effects, mushrooms are also used in the prevention of diseases including cancer, diabetes, stroke, among others (Younis *et al.,* 2014). According to Ferreira and collaborators (2010), in addition to the immunomodulating properties, mushrooms have effective substances for lowering cholesterol, which revert situations of hyperlipidemia, show antithrombotic activity reducing blood pressure, as also hypoglycaemic activity.

The great majority of the studies reporting mushrooms' properties are conducted with the fruiting bodies, but culture media used in mushroom's cultivation have also been explored as potential sources of bioactive compounds (Ma *et al.,* 2016). Otherwise, the *in vitro* culture of mycelia is becoming a promising alternative to obtain sources of bioactive compounds, mainly due to the shorter incubation time and easier culture conditions (less space needed, low probability of contamination and higher biomass yields when compared with fruiting bodies) (Gan *et al*, 2012 and Zhang *et al*., 2016).

The fruiting bodies and mycelia of *A. bisporus* and *P. ostreatus* were previously studied for their chemical composition, antioxidant, anti-inflammatory and cytotoxic activities, after being harvested at different periods (Barros *et al.,* 2009; Reis *et al.,* 2012; Barreira et *al*., 2014; Morro *et al.,* 2012; Ferreira *et al.,* 2010; Martins *et al.,* 2012; Taofiq *et al.,* 2015; Younis *et al.,* 2014; Silva, 2015). However, the present study goes further and compares fruiting bodies, mycelia and culture media in terms of bioactive properties and compounds. Thereby, the main objective was to develop nutraceutical formulations based on fruiting body and mycelium of *A. bisporus* and *P. ostreatus*, as also the corresponding culture media, highlighting the potential of *in vitro* culture as a tool to improve production of bioactive compounds by two different mushroom species.

1.1. Description of the species to be studied

Mushrooms (**Figure 1**) are macrofungi with distinctive and visible fruiting bodies belonging to the phylum Basidiomycota and Ascomycota. Today, mushrooms are greatly appreciated and marketed throughout the world because of its characteristic taste and culinary value, as well as their nutritional properties (Ferreira *et al.,* 2009 and Ferreira *et*

al., 2010).
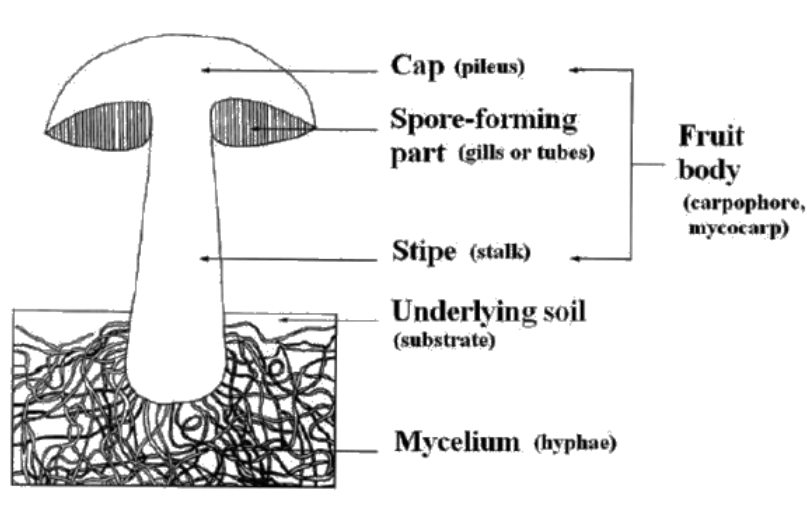
Figure 1: Schematic representation of mushrooms (Kalac, 2000).

Agaricus bisporus L., known as champignon, white mushroom or white button mushroom, belongs to the phylum Basidiomycota. This species has several demonstrated and valuable medicinal properties including anti-tumor, anti-aromatase, antimicrobial, immunomodulatory, anti-inflammatory and antioxidant (Stojkovic *et al*., 2014).

Pleurotus ostreatus (Jacq. Ex Fr.) P. Kumm., better known as oyster mushroom or ''repolgas'', also belongs to the phylum Basidiomycota. It is the edible mushroom most produced all over the world. The production and consumption of Pleurotus is increasing, not only for its easily, quickly growing and organoleptic characteristics, but also for its nutritional value and medicinal properties (Ramos *et al.,* 2011). Its medicinal properties include immunologic, antitumor, anti-inflammatory and antimicrobial activities, among others (Fernandes *et al*., 2015).

1.1.1. *Agaricus bisporus* L.

The consumption of mushrooms is an ancient practice and literature reports its existence since the beginning of civilization. The mushroom *Agaricus bisporus* (**Figure 2**) is the most important fungi among the edible mushrooms cultivated worldwide (Sanchez *et al.,* 2008). With the advancement of technology, today much of the research is directed to the genetic improvement of strains of *A. bisporus*, because these are genetically unstable (Li *et al*., 1994 and Umar & Van Griensven, 1999).

Figure 2: *Agaricus bisporus.*

The mushrooms are gaining worldwide recognition as important functional foods and as sources of therapeutical molecules for the treatment of some diseases (Chang, 2008). In addition to these qualities edible mushrooms are very versatile in cooking, as they can be baked, boiled, grilled or sautéed, and even combined with soups, patés, salads and teas (Brill, 2002).

Beelman and collaborators (2003) published a study related with bioactive compounds in *A. bisporus*, and reported for this species a higer nutritional value but similar bioactive components in comparison with *Lentinus edodes* and *Pleurotus spp*.

A. bisporus has a cap of 5 to 12 cm, convex with curved edge, becoming plane with age, white color, smell and mild taste; it has a pink-brown laminae becoming purple-brown and finally dark brown, a stem of 2 to 5 cm with bulbous base, and dark brown spores (Breitenbach & Kranzlin, 1995 and Sanchez, 2008).

A. bisporus is one of the edible cultivated fungi with higher economic importance. It is a microscopic fungus, with distinctive fruiting body, being large enough to be seen with the "naked eye" and be picked up by hand. It has no cholesterol, and insignificant levels of vitamin A or vitamin C, but it is a source of some B vitamins, in particular, riboflavin (B2), niacin (B3) and pantothenic acid (B5), ergosterol, representing 90% of its sterol fraction (when exposed to ultraviolet light is converted to pro-vitamin D), essential minerals (especially selenium, copper and potassium), phenolic compounds, having also a high amount of crude protein (Barros *et al.,* 2008; Chang, 2008 and Barreira *et al.,* 2014).

A. bisporus (white and brown) exhibit total phenolic contents of 23.34 and 37.33 mg GAE (gallic acid equivalents)/g of extract, respectively (Reis *et al.* 2012b), and a concentration of free ergosterol (analysed by HPLC-UV) of 7.80 mg/g dry matter (Jasinghe, 2005). The taxonomic classification is in the following table (**Table 1**).

Table 1: Taxonomic classification of *Agaricus bisporus* (Braga *et al.,* 1998).

Kingdom:	Fungi
Division:	Basidiomycota
Class:	Homobasidiomycetes
Order:	Agaricales
Family:	Agaricaceae
Genus:	*Agaricus*
Species	*A. bisporus*

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

Among the many existing edible mushrooms, only a few species are used as food and commercially cultivated. The three most cultivated species are *Agaricus brasiliensis, Lentinula edodes* and *Pleurotus ostreatus* (**Figure 3**) (Ribas, 2006), being the genus Pleurotus the third type most produced worldwide (Dias, 2010).

Figure 3: *Pleurotus ostreatus.*

P. ostreatus presents a convex cap plan-convex, grayish or whitish color and with a diameter from 5 to 15 cm. At the bottom of the hat, the blades are arranged radially, with whitish and rather narrow. This species also has a side foot hair and very short compared to the diameter of the cap. The edible part has a whitish color with pleasant and intense fragrance (Coelho, 2012).

Mushrooms of the Pleurotus genus represent a low cost food with high protein content, essential amino acids, higher percentage of unsaturated fatty acids in comparison with saturated fatty acids, various vitamins and minerals, and low levels of nucleic acids, sugars

and calories (Manzi *et al.,* 2001 and Bonatti *et al.,* 2004). Moreover, the advances made in the field of biotechnology allowed the identification of important medicinal properties of mushrooms attributed to various bioactive molecules, such as polysaccharides, ergosterol, phenolic compounds, lecithins and some immunomodulatory amino acids including arginine and glutamine (Viegas *et al*., 2006).

The total phenolic content in *P. ostreatus* is 12.45 mg GAE/g of extract (Reis *et al.,* 2012b) and its concentration in free ergosterol (determined by HPLC- UV) is 4.40 mg/g dry matter (Jasinghe, 2005).

P. ostreatus is a decomposer mushroom of vegetable matter. This species and other species of mushrooms produce extracellular ligninolytic enzymes such as lignin peroxidase, manganese peroxidase and laccase, which are involved in lignin degradation. This feature gives mushrooms the ability to grow on the trunks of living or dead trees (Martinez *et al.,* 2001 and Coelho, 2012). The taxonomic classification of *P. ostreatus* species is presented in the following table (**Table 2**).

Table 2: Taxonomic classification of *Pleurotus ostreatus* (Adapted of Alexopoulos *et al*., 1996).

Kingdom:	Fungi
Division:	Basidiomycota
Class:	Agaricomycetes
Order:	Agaricales
Family:	Pleurotaceae
Genus:	Pleurotus
Species	*P. ostreatus*

1.2. The mushrooms as a source of bioactive compounds

The mushrooms are consumed by people of diverse cultures, both for its gastronomic characteristics, as also medicinal features. However, its use as functional foods is more evident in Eastern cultures, in which the use of mushrooms in health maintenance began thousands of years ago with the Chinese (Chang, 1996).

The mushrooms have a lot of bioactive compounds. Examples of these compounds in mushrooms are tocopherols, phenolic compounds (phenolic acids) (**Table 3**), ergosterol

(**Table 4)**, among others, some of them responsible for their nutraceutical potential (Barros *et al.,* 2008a; Barros *et al.*, 2009 and Ferreira *et al.,* 2009). Nutraceuticals present in mushrooms have been related to their antioxidant, anti-inflammatory and cytotoxic activities (Fernandes *et al.,* 2015).

Table 3: Phenolic acids in mushrooms reported in the literature (A - Barros *et al.,* 2009; B - Muszyńska *et al.*, 2013; C - Reis *et al.,* 2012).

Phenolic acids acid (µg/g dw)						
Species	**Sample**	**Gallicaci d**	**Protocatechui c acid**	***p-*Hydroxybenzoic acid**	***p-*Coumaric acid**	**Cinnamic acid**
Agaricus bisporus	Mushro om	63 ± 13^{C}	Nd	nd	2.31 ± 0.06^{C}	0.38 ± 0.02^{C}
		Nd	Nd	26 ± 2^{A}	nd	8.7 ± 0.7^{A}
	Myceliu m	31 ± 4^{C}	Nd	0.02 ± 0.001^{C}	3.7 ± 0.2^{C}	0.76 ± 0.03^{C}
Pleurotus ostreatus	Mushro om	Nd	0.77 ± 0.02^{C}	1.56 ± 0.06^{C}	0.81 ± 0.03^{C}	0.23 ± 0.02^{C}
		Nd	2.52 ± 0.03^{B}	3.60 ± 0.05^{B}	nd	1.09 ± 0.01^{B}
	Myceliu m	Nd	Nd	0.05 ± 0.0001^{C}	nd	9.7 ± 0.9^{C}

dw- dry weight; n.d. – not detected.

Table 4: Ergosterol content in mushroom fruting bodies reported in the literature (A - Barreira *et al.,* 2014 and B- Villares *et al.,* 2014).

Species	**Ergosterol (mg/100 g dw)**
Agaricus bisporus	352±1 A
	6.4±0.2 B
Pleurotus ostreatus	104±1 A
	3.3 ± 0.2 B

1.2.1. Phenolic acids

Phenolic compounds range from simple molecules up to other with high degree of polymerization (Bravo, 1998 cited by Smith *et al.,* 2002). The presence of these compounds has been highly documented due to its pharmacological and antinutritional activities (Nagem *et al.*, 1992; Gamache *et al*., 1993; Ivanova *et al.,* 1997; Aziz *et al.*, 1998; Fernandez *et al.,* 1998 and Hollman Katan, 1998 cited by Soares, 2002).

The phenolic compounds usually appear as glucosides and esters rather than the free compounds, because of the higher stability of these molecules (Fraga, 2010). According to

Ferreira and collaborators (2009), phenolic compounds are described as biologically active substances having antioxidant, anti-inflammatory or anti-tumor properties. Phenolic molecules are hydroxylated aromatic compounds having in their basic constitution one or more aromatic rings with one or more hydroxyl groups. They include a large number of subclasses, such as phenolic acids, flavonoids, tannins, stilbenes, among others, which indicate a large diversity of chemical structures. The flavonoids are the most widely distributed sub-class. They are found in higher levels in plants and vegetables but mushrooms also have significant amounts of phenolic compounds (Ferreira et al, 2009 and Palacios *et al*, 2011).
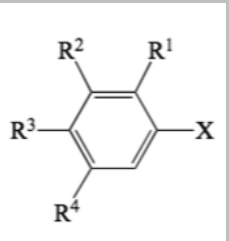
The major phenolic compounds from mushrooms are phenolic acids. Phenolic acids are characterized by having a benzene ring, a carboxyl group and one or more hydroxyl and/or methoxyl groups in the molecule, imparting antioxidant properties both to food as to the organism after ingestion (Crozier *et al.,* 2006 and Fraga, 2010 cited by Carocho & Ferreira, 2013). Phenolic acids are usually found in vegetables and fruits present in our diet, and some of which are among the most bioactive and therapeutically useful substances (Apak *et al*., 2007 cited by Ferreira *et al.,* 2009). According to Soares (2002), phenolic acids, in addition to its presence in their natural form, can also bind to each other or other compounds.

Phenolic acids can be divided into two main groups, hydroxybenzoic acids (**Table 5**) and hydroxycinnamic acids (**Table 6**), which are derivatives of the non-phenolic benzoic and cinnamic acids, respectively. The hydroxybenzoic acid derivatives generally occur in bound form, and are typically components of complex structures such as tannins and hydrolyzable lignins. It can also be found bound to sugars or organic acids in foods, exhibiting seven carbon atoms and being simple phenolic acids found in nature. The derivatives of hydroxycinnamic acid are mainly present in the bound form as structural components of the cell wall, such as cellulose, lignin and proteins, as well as associated with organic acids such as tartaric acid or quinic acid, by ester bonds and exhibit nine carbon atoms, being the structures with seven carbons the most commonly found in the vegetable and fungi kingdoms (Ferreira *et al.,* 2009).

Table 5: Chemical structure of the benzoic acid derivatives found in mushrooms (Ferreira *et al.,* 2009).
* Aldeydes are groups under the corresponding phenolic acid class.
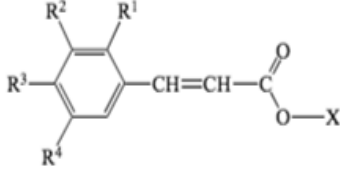
Benzoic acid derivatives	Substitution					
	X	R^1	R^2	R^3	R^4	
p-Hydroxibenzoic	COOH	H	H	H	OH	
Protocatechuic	COOH	H	H	OH	OH	
Gallic	COOH	H	OH	OH	OH	
Gentisic	COOH	OH	H	H	OH	
Homogentisic	CH_2COOH	OH	H	H	OH	
Vanillic	COOH	H	OCH_3	OH	H	
5-Sulphosalicylic	COOH	OH	H	H	HSO_3	
Syringic	COOH	H	OCH_3	OH	OCH_3	
Veratric	COOH	H	OCH_3	OCH_3	H	
Vanillin	CHO*	H	OCH_3	OH	H	

Table 6: Chemical structure of the cinnamic acid derivatives found in mushrooms (Ferreira *et al.,* 2009).
* The carboxylic group is esterified with quinic acid.

Cinnamic acid derivatives	Substitution					
	X	R^1	R^2	R^3	R^4	
p-Coumaric	H	H	H	OH	H	
o-Coumaric	H	OH	H	H	H	
Caffeic	H	H	OH	OH	H	
Ferulic	H	H	CH3 O	OH	H	
Sinapic	CH3O	H	CH3O	OH	CH3O	
3-*O*- caffeoylquinic	*	H	OH	OH	H	
4-*O*- caffeoylquinic	*	H	OH	OH	H	
5-*O*- caffeoylquinic	*	H	OH	OH	H	

1.2.2. Ergosterol

Sterols are special forms of steroids that can be found in animals (zoosterols), plants (phytosterols) and fungi (mycosterols) (Barreira & Ferreira, 2015). They consist of a tetracyclic structure of four rings linked together, consisting of three to six carbon atoms and other ring with five carbon atoms (steroid nucleus) (**Figure 4**) with a hydroxyl group on the C3 position and an aliphatic chain linked to the steroid nucleus (Fahy *et al*., 2005 cited by Diz, 2015).

Ergosterol is the main sterol present in mycelial cells or membranes of most fungi. The highest levels of this compound are in phospholipid layer of the membrane where fungal ergosterol plays an important structural and hormonal function in cell cycle progression (Ricardo, 2015).
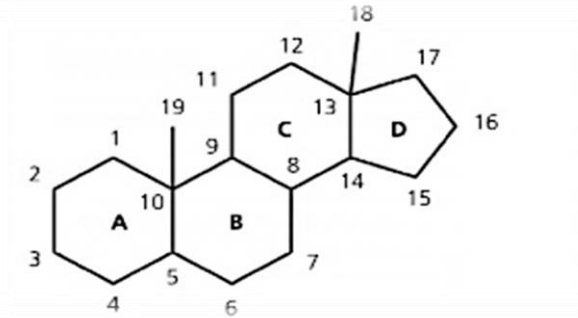
Figure 4: Steroid nucleus (Barreira & Ferreira, 2015).

The ergosterol is abundant in edible mushrooms (Kuo *et al.,* 2011). According Jasinghe and Perera (2005), the nature of ergosterol varies depending on the mushroom species, within the same species, with different varieties of mushrooms and with the maturity stage. These authors indicate that the concentration of sterols in mushrooms, especially ergosterol, depends on the part of the mushroom tissue used, maturity stage and growth conditions. Savón and collaborators (2002) cited by Diz (2015) also reported that, in relation to specific genetic traits, ergosterol content in mushrooms also depends on environmental factors such as light, heat, temperature, humidity and the type of substrate used on the growth of mushrooms.

The ergosterol is present in two basic forms, namely, as free ergosterol and esterified ergosterol. The relative abundances of free and esterified ergosterol are different according with the species. The free ergosterol is most important for the integrity of the cell and contributes to various cellular functions. The ergosterol ester, kept in cytosolic lipid particles, is a fixed storage form of sterol and can serve as an intermediate for the ergosterol functioning (Barreira & Ferreira, 2015).

According to Diz (2015), ergosterol is a molecule with high commercial value and is the main sterol in fungi. For example, it is the most abundant mycosterol in *Agaricus bisporus*, and is also found in corn oils, cottonseed, groundnut and linseed (Lagarda *et al.,* 2006 cited by Barreira & Ferreira, 2015). Zymosterol, ergosta- 5,7-dienol, 24-methyl cholesterol, and methylene cholesterol are also important examples of sterols in fungi (Mattila *et al*., 2002 and Christie, 2013 cited by Barreira & Ferreira, 2015).

The ergosterol or provitamin D2, whose chemical name is 5,7,22-ergostatrien-3β-ol has the empirical formula $C_{28}H_{44}O$ and the relative molecular mass of 396.36 g/mol. The ergosterol compound is solid, crystalline and colorless, and has a melting point in the range

of 161-166 ºC. In vacuum, it supports the temperature of 250 ºC without decomposition (Barreira *et al.,* 2014 and Ferreira, 1985 cited by Ricardo, 2015).

A metabolite, which is commonly found in mushrooms, is ergosterol peroxide, having become a very promising compound for health and significant pharmacological activities, such as antioxidant, antimicrobial and antitumor. In addition, these compounds reduce the incidence of cardiovascular disease and pain associated with inflammation by inhibiting the cyclooxygenase enzyme (Yuan *et al.,* 2007).

The antitumor activity of ergosterol may be due to its inhibitory capacity of angiogenesis (development of new blood vessels from existing ones) induced by solid tumors (Takaku *et al.*, 2001; Zaidman *et al.*, 2005; Slominski *et al.,* 2005 cited by Ricardo, 2015).

Other studies have indicated that free ergosterol (the main component of the lipid portion) was responsible for the antioxidant activity of the hexane extract of *Agaricus bisporus* brown mushroom (positive correlation between ergosterol content and antioxidant activity; r2 > 0.89) (Shao *et al.,* 2010). Furthermore, ergosterol may exhibit hypocholesterolemic properties, since it is able to reduce cholesterol levels in the serum (Gil-Ramirez *et al.*, 2013).

1.3. Bioactivity of extracts rich in phenolic acids and ergosterol

Edible 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. They are appreciated throughout the world not only for its organoleptic characteristics but also for its nutritional and functional properties (Ferreira *et al.*, 2010).

The presence of bioactive compounds, namely ergosterol and phenolic compounds, could explain their antioxidant, anti-inflammatory, cytotoxic and antimicrobial properties (Ricardo, 2013).

1.3.1. Antioxidant activity

In general, the term antioxidant is used for sorting molecules which are present in low quantities in the substrate that is oxidizable and which reacts rapidly in order to suppress or prevent oxidation (Magalhães, 2009).

Maintaining the balance between the production of free radicals and antioxidant defenses

(enzymes and non-enzymatic molecules) is a prerequisite for the normal functioning of the body. When this equilibrium tends to uncontrolled production of free radicals means that the body is in oxidative stress and in such situations, excess free radicals can oxidize and damage cell lipids, proteins, DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), inhibiting their normal function, leading to various diseases such as atherosclerosis, diabetes, cirrhosis, cataracts, premature aging and cancer (Valko *et al.,* 2007 and Ferreira *et al.*, 2009).

According Ferreira and Abreu (2007), the exposure of organisms to free radicals, led to the development of endogenous defense mechanisms to eliminate them. But despite all organisms possess defense and repair systems that have evolved to protect them against oxidative damage, these systems are often insufficient to prevent completely, the damage induced by oxidative stress (Yang *et al*., 2001). Nevertheless, the presence of antioxidants in the diet can help the endogenous defense system, reducing oxidative damage (Fang *et al*., 2002; Liu, 2003 and Barros *et al*., 2008b).

Currently, antioxidants have a range of applications, from food to pharmaceutical industry, as also plastic's industry and lubricating oils. The best known synthetic antioxidants are BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), TBHQ (tert-butylhydroquinone) and PG (propyl gallate), which are widely used in both food and pharmaceutical industry. However, BHA and BHT have been restricted in their use due to possible toxic and carcinogenic effects (Silva, 2015). Thus, the use of antioxidants from natural sources such as mushrooms, assumes increasing importance.

This antioxidant activity in mushrooms is related mainly to its high content of phenolic compounds, are recognized as excellent antioxidants because of their ability to block free radical by transfer of a single electron and excellent redox properties of the phenolic hydroxyl groups (Cheung, 2008; Kim *et al*, 2008; Jayakumar *et al,* 2009 cited by Ricardo 2013).

Recent studies indicate a direct relationship between the content of phenolic compounds and antioxidant activity of several species of mushrooms. A study performed with methanol extracts of some cultivated species of edible mushrooms from Portuguese origin, such as *Agaricus bisporus* (white), *Agaricus bisporus* (brown), *Pleurotus ostreatus*, *Pleurotus eryngii* and *Lentinula edodes* revealed that these species have a scavenging effect on DPPH• (2,2-diphenyl-1-picrylhydrazyl) radicals, inhibit lipid peroxidation and have a strong reducing power (**Table 7**). (Reis *et al*., 2012b).

Agaricus bisporus and *Pleurotus ostreatus*, as well as other species of cultivated edible mushrooms are a good source of natural antioxidants, presenting in its constitution phenolic compounds, especially phenolic acids, followed by tocopherols (mainly α-tocopherol), ascorbic acid and carotenoids, especially the β-carotene (Barros *et al*., 2009 and Ferreira *et al.,* 2009). According to Rodriguez-Vaquero and collaborators (2007) and Karaman and collaborators (2010), the strongest antioxidant properties and capability of cell protection against hydrogen peroxide was evident for vanillic acid, and among cinnamic acid derivatives, for caffeic acid. *p*-Hydroxybenzoic, gallic and protocatechuic acids found in mushrooms are characterized by antioxidant, antibacterial, antiviral, antifungal, anti-inflammatory and gastric secretion-stimulatory actions, documented by *in vitro* and *in vivo* studies. However, according to Heleno and collaborators (2015) little is known about the bioactive phenolic forms *in vivo*.

Studies have shown that ergosterol and its peroxidation products may also contribute to potential health benefits and significant pharmacological activities, namely antioxidant activity (Yuan *et al*., 2007).

Table 7: Reducing power, scavenging activity and lipid peroxidation inhibition of the studied edible mushrooms according with Reis *et al.* (2012b).

Species	Sample	Reducing power	Scavenging	Lipid peroxidation inhibition	
		Ferricyanide/Prussian blue assay (EC$_{50}$; mg/mL)	DPPH scavenging activity assay (EC$_{50}$; mg/mL)	β-carotene/ linoleate assay (EC$_{50}$; mg/mL)	TBARS assay (EC$_{50}$; mg/mL)
Agaricus bisporus	Mushroom	1.80 ± 0.03	3.13 ± 0.09	3.4 ± 0.1	2.9 ± 0.5
		3.63 ± 0.02[F]	9.61 ± 0.07[F]	21.4 ± 0.5[F]	
	Mycellium	8.12 ± 0.06	40 ± 2	2.4 ± 0.6	0.87 ± 0.76
Pleurotus ostreatus	Mushroom	3.31 ± 0.03	6.5 ± 0.2	2.7 ± 0.2	2.6 ± 0.9
			5.1 ± 0.7[G]	440 ± 43[G]	
	Mycellium	4.7 ± 0.2	58 ± 3	17 ± 2	1.0 ± 0.3
			29 ± 6[G]	392 ± 51[G]	

Concerning Folin-Ciocalteu assay, higher values mean higher reducing pawer: for the other assays, the results are presented in EC$_{50}$ values, what means that higher values correspond to lower reducing power or antioxidant potential. EC$_{50}$: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance for the Ferricyanide/Prussian blue assay. [F] Martins *et al*., 2012. [G] Silva, 2015.

1.3.2. Anti-inflammatory activity

Inflammation is considered part of the complex biological response to eliminate pathogens, damaged cells, or irritation. This response leads to many physical symptoms, such as fever, pain, swelling, etc. When cells are exposed to immune stimulants, pro-inflammatory cells such as macrophages, or other host cells start to produce cytokines and other mediators that

initiate the process of inflammation (Taofiq *et al.,* 2015).

Among the various inflammatory mediators, the most common are the interleukins (IL-1β, IL-6, IL-8), tumor necrosis factor (TNF-α), nuclear-kB factor (NF-kB), intercellular adhesion molecule 1 (ICAM-1) inducible type of cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), 5-lipoxygenase (5-LOX), and the inducible nitric oxide synthase (iNOS), which leads to the production of reactive nitrogen species such as nitric oxide (NO). Over-production of these inflammatory mediators leads to different types of cellular damage (Kanwar *et al*., 2009 cited by Taofiq *et al.,* 2015).

Nowadays, NSAIDs are generally administered to reduce inflammation in the body. But long-term administration of NSAIDs has significant side effects on the gastrointestinal tract (Smalley *et al*., 1995 and Sinha *et al.,* 2013), as well as other serious complications such as hypertension and cardiovascular toxicity, etc. (Dugowson & Gnanashanmugam, 2006 and Meek *et al*., 2010).

Many bioactive compounds present in mushrooms exhibit significant anti-inflammatory properties (**Table 8**), based on their ability to reduce the production of inflammatory mediators (Ferreira *et al.,* 2009; Elsayed *et al.,* 2014 and Heleno *et al.,* 2015). Several compounds have been identified as potential natural and safe drugs, without the harmful side effects characteristic of NSAIDs. These compounds are responsible for anti-inflammatory activity, and include mostly beta-glucans, triterpenoids, glycoproteins and phenolic compounds (Taofiq *et al.*, 2015).

According to Yuan and collaborators (2007) the products of ergosterol peroxidation reduce pain associated with inflammation by inhibiting the enzyme cyclooxygenase.

As previously demonstrated, the edible white button mushroom (*Agaricus bisporus*) enhanced NK cell activity in mice through the increased production of IFN-δ which induced maturation of dendritic cells, and TNF-α, increasing the production of IL-12 (Wu *et al.,* 2007 and Ren *et al.,* 2008).

Oyster mushrooms (Pleurotus species) belong to the world of consumed mushrooms that, in addition to their nutritional value, demonstrate health-promoting (antioxidant, anti-atherosclerotic, anticancer and immunomodulatory) effects (Jayakumar *et al.,* 2008). Glycosphingolipid when isolated from *Pleurotus ostreatus*, inhibited leukocyte migration to acetic acid-injured tissues (Smiderle *et al.,* 2008).

Table 8: Some previous studies on anti-inflammatory activity of the two studied mushroom species.

Species	Inhibition of NO production	References
Agaricus bisporus	30% at 0.5 mg/mL	Moro *et al.,* (2012)
Pleurotus ostreatus	15% at 0.5 mg/mL	
Extract concentrations responsible for 50% of reduction of NO production (EC$_{50}$ values) in RAW 264.7 cell line		
Species	**EC$_{50}$ values (µg/mL)**	**References**
Agaricus bisporus	190 ± 6	Taofiq *et al.,* (2015)
Pleurotus ostreatus	96 ± 1	

1.3.3. Cytotoxic activity

Cancer is a disease characterized by a cell population, which grow and divide without respect to normal limits, invade and destroy adjacent tissues. But, the cells have enzyme systems capable of repairing the majority of such damages. Although these systems are efficient, some injury or even spontaneous errors during DNA replication are not repaired (Kellof & Sigman 2000).

The causes of cancer are diverse, complex, and only partially understood. Cancers are primarily an environmental diseases with 90–95% of cases attributed to environmental factors and 5–10% due to genetics common environmental factors that contribute to cancer death including tobacco (25–30%), diet and obesity (30–35%), infections (15–20%), radiation (both ionizing and non-ionizing, up to 10%), stress, lack of physical activity, and environmental pollutants. Cancer causing viral infections such as HBV/HCV and HPV are responsible for up to 20% of cancer deaths in low and middle-income countries (Younis *et al.,* 2014).

There are different possibilities to cancer treatment including surgery, chemotherapy, radiation therapy, and palliative care. These treatments are used depending on the type, location and grade of the cancer as well as the person's health and wishes. Treatment options are often expensive and have side effects. For example, most chemotherapeutic agents for the treatment of cancer destroy tumors and arrest cancer progress but also damage healthy cells and tissues This situation has forced scientists to search for new antitumor substances from various natural sources to develop more effective and safer agents to inhibit the growth of cancer cells, and not causing harm to the host (Ajith &

Janardhanan, 2003).

Popovic and collaborators (2013) defines mycotherapy as the study of the use of extracts and compounds derived from mushrooms as medicines or health promoting agents. Mycotherapy of cancer is a scientific field relatively new and promising, which deals with anticancer agents derived from mushrooms.

Identification of active ingredients in the extracts, in other words, the isolation of new anti-tumor mushroom substances has become a matter of great importance, in view of the complexity and distribution of various types of cancer in the world population (Zong *et al*., 2012).

A wide variety of compounds and complex fractions have been isolated and/or purified from some edible mushrooms, with particular importance in relation to anticancer and cancer preventive activity (**Table 9).** These activities are attributed mainly to polysaccharides, sterols, phenolic compounds etc. (Takaku *et al*., 2001; Liang *et al.,* 2011; Vaz *et al*., 2012 and Zong *et al.,* 2012).

Some species of the genera Pleurotus, Agaricus, Flammulina, Ganoderma, among others are medicinally valuable due to its anti-tumor activity **(Table 9)** (Ferreira *et al*., 2009 and Gunawardena *et al*., 2013 cited by Younis *et al*., 2014).

According to Song and collaborators (2009), Ma and collaborators (2013) (cited by Popovic *et al.,* 2013), various derivatives of ergosterol have been isolated from mushrooms, for example, ergosterol peroxide and trametenolic acid, and showed cytotoxicity in prostate cell lines and human breast carcinoma.

According to Takaku and collaborators (2001), oral administration of ergosterol (400 and 800 mg/kg for 20 days) to mice with Sarcoma 180, reduced significantly the tumor growth without any side effects. Other studies showed that ergosterol peroxide induced death of miR-378 transfected cells (Wu *et al.,* 2012), and ergosterol peroxide and trametenolic acid isolated from *Inonotus obliquus* exerted cytotoxic activity in prostate cell lines and human breast carcinoma (Ma *et al*., 2013).

Phenolic compounds isolated from mushrooms also showed high cytotoxic activity against human tumor lines cells. Protocatechuic acid (phenolic acid) and a related compound (cinnamic acid), induced significant inhibition of cell growth of a human non-small lung carcinoma cell line (NCI H460); the effect of cinnamic acid was the most pronounced (Vaz *et al.* 2012).

Table 9: Compounds with antitumor potential found in the studied mushroom species (Ferreira *et al.,* 2010) and cytotoxic activity of extracts obtained from the same speices against the two carcinoma cell lines (Younis *et al.,* 2014).

Mushroom Species	Antitumor Agents	References
Agaricus bisporus	490 Quinone (-L-glutaminyl-4-hydroxy-2,5-benzoquinone)	Zaidman et al. (2005)
	Selenium	Tiffany et al. (1978)
	Lectins	Wang et al. (1998)
Pleurotus ostreatus	Selenium	Zaidman et al. (2005)
	Pleuran	Bobek & Galbavy (2001)

Mushroom Species	Sample	HepG2		HeLa	
		Maximum inhibitory %	IC$_{50}$ (µg/mL)	Maximum inhibitory %	IC$_{50}$ (µg/mL)
Agaricus bisporus	Fruiting bodies	66.6±3.2	30.2±2.2	71.3±1.9	28.8±1.8
	Mycelia	41.1±2.1	54.9±2.4	57.4±2.9	38.7±1.9
Pleurotus ostreatus	Fruiting bodies	70.4±2.8	30.4±1.9	70.9±2.7	26.5±1.8
	Mycelia	40.8±2.6	56.3±2.5	45.2±1.5	52.2±2.5

IC$_{50}$: Extract concentration corresponding to 50% of cytotoxic activity.

1.4. *In vitro* culture as a tool to improve the production of bioactive compounds

The *in vitro* culture aims to clarify the optimum conditions for fungal growth, regarding culture medium, temperature and time of incubation and this knowledge is a prerequisite for commercial cultivation in formulated substrate (Hatvani, 2001). *In vitro* culture emerges as a biotechnological tool feasible for the production of bioactive compounds that can be used in several areas, and particularly in order to make additional efforts for the sustainable conservation and rational use of biodiversity (Karuppusamy, 2009 cited by Dias *et al.,* 2016).

1.4.1. Advantages and disadvantages of *in vitro* culture

According to Peter and collaborators (2005) and Kolene and collaborators (2008) (cited by Dias *et al.,* 2016) the World Health Organization (WHO) estimates that 80% of the population still depends today on traditional medicine for primary health care. Furthermore, many of the anticancer and anti-infeccious drugs available on the market are derived from natural products. Within the requirement of uninterrupted market naturally derived products faces an environmental concern regarding the loss of natural populations, genetic diversity, habitat degradation and even species extinction (Roberto & Francesca, 2011).

In 1994, the United Nations Food and Agriculture Organization (FAO) has authorized the use of *in vitro* culture techniques as a method for the production of food natural compounds (Anand, 2010 and Roberto & Francesca, 2011). In 2002, FAO published a report in association with the IAEA (International Atomic Energy Agency - Division of Nuclear Techniques in Food and Agriculture) referring to the theme of *in vitro* culture techniques for the production of bioactive compounds as having a high value, indicating how it can be carried out more economically by researchers and industry (FAO/IAEA, 2002).

The cells of species grown *in vitro* synthesize, accumulate and sometimes exude many classes of metabolites. The bioactive compounds are of particular interest, and much effort has been devoted to getting some of the most precious and therapeutic properties (Vanisree & Tsay, 2004).

Murthy and collaborators (2015) developed a safety assessment of food ingredients obtained by *in vitro* culture and proposed some protocols for evaluating the toxicity of these compounds and also its potential bioactivity. The *in vitro* culture includes handling under aseptic conditions and in case of plant culture, it must be carried out in a medium under controlled conditions of light, humidity and temperature (Smetanka 2008 cited by Dias *et al*., 2016). This controlled production system allows increased uniformity and standardization of extracts, such as the concentration of the desired compounds, maintaining the same genetic characteristics of the best clones for production (Chaturvedi *et al*., 2007 Cited by Dias *et al*., 2016).

The combination of the engineering process, biotechnology and biochemistry led to a significant improvement in production yields, especially for the *in vitro* culture as the preferred method for the production of bioactive compounds (Zhou & Wu, 2006 and Kolewe *et al*., 2008 cited by Dias *et al*., 2016).

Many advantages are associated with the *in vitro* production of bioactive compounds, for example: control and optimization of production conditions; low probability of contamination; preservation of the microorganism for additional studies (Black, 1997); control of the final product; selection of the best clones by genetic engineering; producing pure compounds; improving the nutritional effect of the produced species; reduction of undesired compounds; free production of herbicides and pesticides; chemical synthesis of the novel compounds; production is not dependent on climatic and geographical conditions (Verpoorte *et al*., 1999 and Chattopadhyay *et al.,* 2002 cited by Dias *et al.,* 2016).

However, according to Anand (2010), the most important advantage of *in vitro* culture is its ability to provide continuous, sustainable, economic and viable production of natural compounds, regardless of the geoclimatic conditions and a highly controlled environment.

The *in vitro* culture, as any other process is sensitive to some problems of environmental or biological order, which affect directly the development of the cultures. Among these problems can be mentioned: low rate of development; it may happen a partial or complete loss of culture. Moreover, if the production on a large scale requires costly equipment, the procedure requires special care and careful observation; there may be error in the identity of the organisms after the culture; risk of infection, if precautions are not taken; risks of genetic variation (Engelmann, 1991; Santos *et al.,* 2001 and Ahmed *et al*., 2008).

1.4.2. Mycelium production

The mycelium is the mass of hyphae that makes up the vegetative body of a mushroom. Colonies of mushrooms comprised of mycelium are found on the ground and in many other substrates. A single spore germinates in a monokaryotic mycelium, which cannot sexually reproduce; when two compatible monokaryotic mycelia join and form a dikaryotic mycelium, this mycelium can form fruiting bodies such as mushrooms (Leiva *et al.,* 2015).

The mycelial growth for a period of time has several stages with typical physiological properties (Montini *et al.,* 2006). The extent of this growth can be done in different ways, namely radial growth, vigor, growth rate and weight of the mycelium. Under experimental conditions, the use of solid culture medium for evaluation of fungal growth is considered appropriate because, in nature, the fungi are developed commonly on solid substrates, such as plant debris, animals or the soil (Bononi *et al.,* 1999). Savoie and collaborators (1995) recommended the use of a composition of culture medium similar to growth substrate.

The inoculum is obtained by growing the cultivated mushroom's mycelium in Petri dishes or flasks. In order to inoculate the mycelium, the inoculum must be created in an artificial culture medium in a lab. Cultures on agar and in liquid media (entirely artificial systems) are excellent models and are indispensable for mycelium development. It must be pointed out that the medium used to grow the mycelium is often specific to a certain mushroom species. Mycelium growth is strongly influenced by the *in vitro* conditions, such as the

period of incubation, temperature, pH, size of the inoculum, etc. (Leiva *et al.,* 2015).

The mycelia mushrooms are usually isolated from sporocarps on solid or liquid media (Melin-Norkrans (MMN), PDA medium (Potato Dextrose Agar) or PDB (Potato Dextrose Broth), or others, according to nutrients requirements of the species). The strains are maintained on Petri dishes (9 cm diameter) containing the same media mentioned above at 25°C in the dark and subcultured. After growth, the mycelium is recovered from the medium (Reis *et al*., 2011 and Carocho *et al*., 2012).

As mentioned in the previous sections, mushrooms mycelia have demonstrated several bioactive properties, being used in the treatment, but mostly in the prevention of different diseases. Therefore, with the market requirements for these bioactive compounds, *in vitro* culture arises as the promising alternative for their production.

1.5 Objectives

As mentioned before, mushrooms have been used for many years by different cultures as teas and nutritional foods, because of their special taste and texture. *Agaricus bisporus* and *Pleurotus ostreatus* are traditionally used as food, and accumulate a large variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids, with great bioactive potential. These species in particular have antioxidant, anti-inflammatory and cytotoxic activities. Although many people appreciate mushrooms there are still some people that do not like its texture and flavor, being interesting to develop nutraceutical formulations that can be used *per si* or incorporated into other foods, providing bioactive properties. On the other hand, the *in vitro* production of mycelium can help to overcome difficulties associated with mushroom's availability issues related to seasonality or adverse climatic conditions, for example.

Therefore, this work had the main objective of developing nutraceutical formulations based on mycelium of *Agaricus bisporus* and *Pleurotus ostreatus*, and will include the following steps:

1) Production of *Agaricus bisporus* and *Pleurotus ostreatus* mycelia to obtain extracts enriched in bioactive compounds namely, phenolic acids and mycosterols. Mycelia from each of the mushrooms were isolated in *i)* solid modified Melin-Norkrans medium (MMN) and liquid medium with the same composition, but without the

agar; *ii)* Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) media, after properly monitorization of the growth.

2) Chemical characterization of the mycelia obtained in terms of phenolic acids (HPLC-DAD) and mycosterols (mainly ergosterol, HPLC-UV).

3) Evaluation of their bioactive properties in particular antioxidant (free radicals scavenging activity, reducing power and lipid peroxidation inhibition), anti-inflammatory (inhibition of NO production in mice macrophages) and cytotoxic (inhibition of the growth of different human tumor cell lines and of primary culture of non-tumor porcine liver cells).

CHAPTER 2.

2. MATERIAL AND METHODOS

2.1. Standards and reagents

The solvents acetonitrile 99.9% and methanol were of high-performance liquid chromatography (HPLC) grade from Lab-Scan (Lisbon, Portugal). The standards of ergosterol, phenolic compounds (gallic, protocatechuic; *p*-hydroxybenzoic, *p*-coumaric, and cinnamic acids) and trolox (6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid) 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), lipopolysaccharide (LPS) 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", Salisburg, UK), and DMEM medium from HyClone (GE Healthcare Life Sciences, Logan, UT, USA). 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).

2.2. Samples and mycelium production

Agaricus bisporus (J.E.Lange) Imbach was bought in the Clube Minipreço store in Braganca and *Pleurotus ostreatus* (Jacq. ex Fr.) P.Kumm. was acquired from a sterile wheat grain and boiled wheat straw cultivation bale in Bragança (**Figure 5**).

Figure 5: *Pleurotus ostreatus* in bales.

Mycelia from both species were produced in the Biology and Biotechnology Laboratory of School of Agriculture of Bragança.

Mycelia from each of the mushrooms were isolated from sporocarps in two solid and liquid media: 1) modified Melin-Norkrans solid medium (MMN) pH 6.6 (NaCl 0.025 g/L; $(NH_4)_2HPO_4$ 0.25 g/L, KH_2PO_4 0.509 g/L; $FeCl_3$ 0.0050 g/L, $CaCl_2$ 0.050 g/L, $MgSO_4.7H_2O$ 0.15 g/L, thiamine 100.10 g/L, glucose 10 g/L, agar 20 g/L and tap water) (Marx, 1969 cited by Reis et al., 2011 and Carocho et al., 2012); 2) MMN liquid medium (prepared using the above reagents, without agar); 3) Potato Dextrose Agar solid medium (PDA) and 4) Potato Dextrose Broth liquid medium (PDB).

The strains were maintained on Petri dishes (9 cm diameter) containing 10 mL of solid media and flasks with 20 mL of the same medium mentioned above at 25 °C in the dark and subcultured until mycelium covered most of the medium: approximately 21 days for *P. ostreatus* in solid medium and 42 days in liquid medium for *A. bisporus*, 56 days in solid and liquid medium. Radial growth measurements were registered every week from the inoculation time until the full growth of the mycelium (covering all available area) was obtained (Reis *et al.,* 2011 and Carocho *et al.,* 2012).

After different growth time's mycelia were recovered from both solid and liquid media, by scraping with a scalpel and sieved (particle size, 2 mm), respectively, in order to remove all medium residues (**Figure 6**).

Figure 6: Illustration of the mycelium production.

Afterwards, all the samples (mycelium, culture medium and fruiting body) were placed in beakers and weighed separately to obtain fresh weight (fw) (**Figure 7**). Subsequently, the samples were frozen and lyophilized (freeze 4.5 FreeZone model 7750031, Labconco, Kansas, USA) to obtain the corresponding dry weight (dw). Finally, they were reduced to a fine powder (20 mesh).

Figure 7: Illustration of mycelia separation from the medium.

2.3. Preparation of the extracts

Dried powder of each sample was placed in a beaker (≈2 g) and was extracted by magnetic stirring with methanol (30 mL) at 25°C and 150 rpm, for 1 h. The extract was separated

from the residue by filtration through Whatman paper No. 4 into a round flask. The residue was re-extracted once more under the same conditions and the filtrates were combined and concentrated with a rotary evaporated at ≈40°C (rotary evaporator, Büchi, Flawil Switzerland) (**Figure 8**). The extraction yield was calculated by measuring the extract weight in relation to the initial mass sample and stock solutions were prepared for the different biological assay measured.
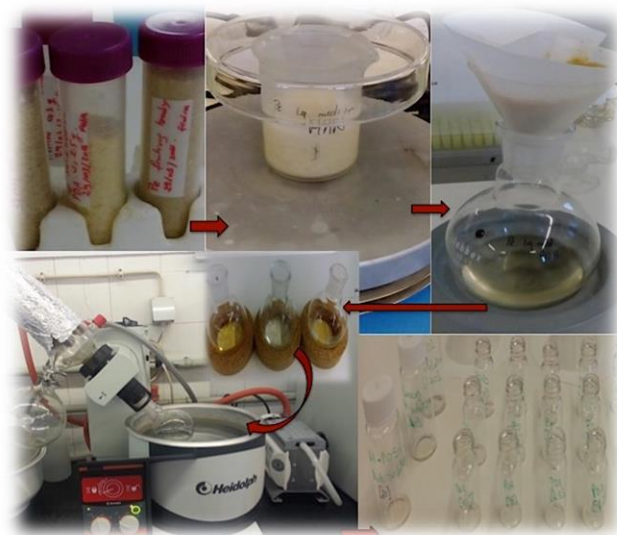
Figure 8: Illustration of the extraction procedure.

2.4. Chemical characterization

2.4.1. In phenolic acids

Each sample was placed in a beaker (≈1g) and was extracted by magnetic stirring with methanol:water 80:20 (v/v) at 25°C and 150 rpm, for 1 h. The extract was separated from the residue by filtration through Whatman paper No. 4 to a round flask. The residue was re-extracted once more and the filtrate was rotary evaporated at ≈40°C to remove methanol (rotary evaporator, Büchi, Flawil Switzerland) (Reis *et al.,* 2012). The aqueous phase was washed with n-hexane, and then submitted to a liquid-liquid extraction with diethyl ether (3 x 50 mL) and ethyl acetate (3 x 50 mL). The organic phases were evaporated at 30 ℃ to dryness, redissolved in water:methanol (80:20), and filtered through a 0.22 µm disposable LC filter disk for HPLC analysis.

The analysis was performed using a Shimadzu 20A series ultra-fast liquid chromatograph (UFLC, Shimadzu Coperation, Kyoto, Japan) as previously described by Reis et al., (2012)

(**Figure 9**). Separation was achieved on a Waters Spherisorb S3 ODS2 C_{18} column (3 μm, 150 x 4.6 mm) thermostatted at 35ºC. 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 9: HPLC-DAD equipment used in the analysis of phenolic acids.

2.4.2. In ergosterol

The extracts prepared above in section 2.3 were dissolved in methanol at a concentration of 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 10**). The components of the HPLC-UV integrated system include a pump (Knauer, Smartline system 1000, 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. The results were expressed in mg per g of extract (Heleno *et al.,* 2016).

Figure 10: HPLC- UV system used in the analysis of ergosterol.

2.5. Evaluation of bioactive properties

2.5.1. Antioxidant activity

The final extracts obtained in section 2.3, were dissolved in methanol obtaining stock solutions (20-80 mg/mL), then subjected to serial dilutions (40 and 0.01953 mg/mL), depending on the stock solutions and according to the assay. The in vitro antioxidant activity of the extracts was evaluated by performing 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 (Fernandes, 2010).

2.5.1.1. DPPH radical scavenging activity

This methodology was performed using a Microplate Reader ELX800 (Bio-Tek Instruments, Inc., Winooski, VT, USA). 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 in each of the 96 wells consisted of different solutions of the extracts (30 µL) to which was added a methanolic solution (270 µL) containing DPPH radical scavenging ($6 \times 10{-5}$ mol/L). The mixture was allowed to stand for 60 min in the dark. The reduction of DPPH radical was determined by measuring the absorbance at 515 nm (**Figure 11).** The radical scavenging activity (RSA) was calculated as percentage of discoloration of DPPH solution using the formula: % RSA = [(ADPPH - AS) / ADPPH] × 100 where AS is the absorbance of the solution in the presence of a given extract concentration and ADPPH is the absorbance of DPPH solution. The extract concentration which leads to 50% radical scavenging activity (EC_{50}) was calculated from the RSA percentage graph as a function of extract concentration. Trolox was used as standard (Fernandes, 2010).
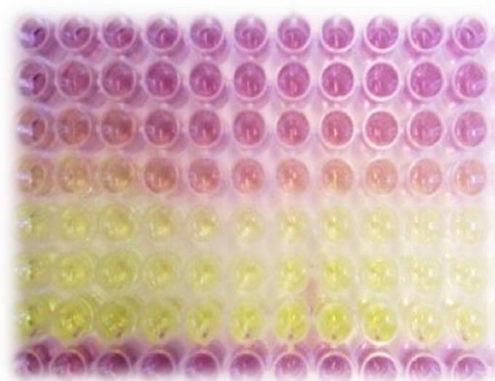
Figure 11: Microplate used in the evaluation of DPPH radical-scavenging activity.

2.5.1.2. Reducing power

This methodology was performed using the Microplate Reader, and 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 (Pinela, 2012).

Different concentrations of the extracts (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) with the addition of potassium ferricyanide (1% w/v 0.5 ml) in eppendorf (2 mL) and incubated at 50 ºC for 20 min. After this incubation time 0.5 mL of trichloroacetic acid 10% were placed in the eppendorf. The mixture (0.8 mL) was placed in 48 wells microplates with deionized water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL) (**Figure 12**), measuring the absorbance at 690 nm. The concentration of extract that provides 0.5 absorbance (EC_{50}) was calculated from the absorbance at 690

nm graph as a function of extract concentration. Trolox was used as standard (Fernandes, 2010).
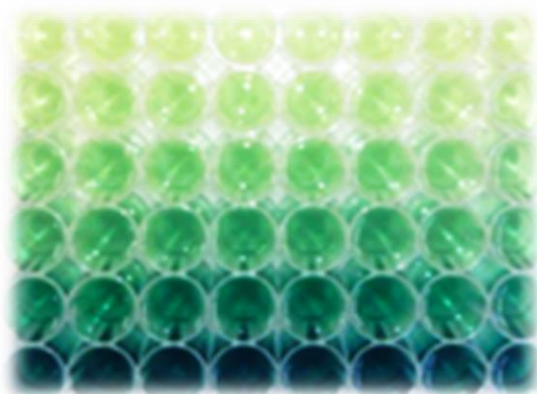
Figure 12: Microplate used in the evaluation of reducing power.

2.5.1.3. Inhibition of discoloration of *β*-carotene

This assay is based on the capacity of the antioxidants to neutralize the linoleate free radical. This neutralization is detected by the discoloration of the yellowish color of *β*-carotene. A solution of *β*-carotene was prepared by dissolving *β*-carotene (2 mg) in chloroform (10 mL). Two mililiters of this solution was transferred into a round bottom flask (100 ml). After the chloroform was removed at 40 ℃ under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing different concentrations of the samples (0.2 mL). The tubes were shaken and incubated at 50 ℃ in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm in a spectrophotometer (AnalytikJena, Jena, Germany) (**Figure 13**). *β*-Carotene bleaching inhibition was calculated using the following equation: (Abs after 2h of assay/initial Abs) × 100. The extract concentration providing 50% of antioxidant activity (EC_{50}) was calculated from the graph of *β*-carotene bleaching inhibition against extract concentrations. Trolox was used as a standard (Fernandes, 2010).

Figure 13: Test tubes used in the β-carotene/linoleate assay.

2.5.1.4. Inhibition of lipid peroxidation in the presence of thiobarbituric reactive substances (TBARS)

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).

Porcine (*Sus scrofa*) brains were obtained from official slaughtering animals, dissected, and homogenized with a Polytron in ice-cold Tris–HCl buffer (20 mM, pH 7.4) to produce a 1:2 (*w/v*) brain tissue homogenate, which was centrifuged at $3000g$ for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with the different solution concentrations (0.2 mL) in the presence of $FeSO_4$ (10 μM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28 % *w/v*, 0.5 mL), followed by thiobarbituric acid (TBA, 2 %, *w/v*, 0.38 mL), and the mixture was then heated at 80 ºC for 20 min. After centrifugation at $3000g$ for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm (**Figure 14**).

The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = $[(A - B)/A] \times 100$ %, where A and B were the absorbance of the control and the compound solution, respectively. The extract concentration providing 50% of antioxidant activity (EC_{50}) was calculated from the graph of TBARS formation inhibition against extract concentrations. Trolox was used as a standard (Fernandes, 2010).
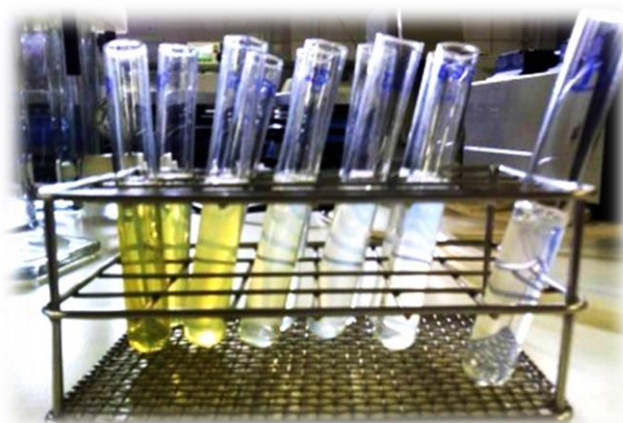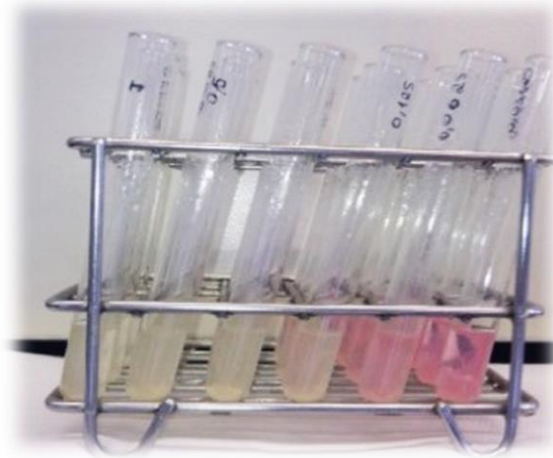
Figure 14: Test tubes used in the TBARS assay.

2.5.2. Anti-inflammatory activity

For the anti-inflammatory activity assay, the methanolic extracts (section 2.3) were dissolved in water at a concentrated of 10 mg/mL. For the various assays, the extracts were then submitted to further dilutions from 10 mg/mL to 0.16 mg/mL. Dexametazona was used as positive control (Taofiq, 2015).

2.5.2.1. Cells treatment

The mouse macrophage-like cell line RAW 264.7 was cultured in DMEM medium supplemented with 10% heat-inactivated foetal bovine serum, glutamine and antibiotics at 37 °C under 5% CO_2, in humidified air. For each experiment, cells were detached with a cell scraper. In the experiment cell density of 5×10^5 cells/mL was used, and the proportion of dead cells was less than 5% according to the Trypan blue dye exclusion test. Cells were seeded in 96-well plates at 150,000 cells/well and allowed do attach to the plate overnight. Subsequently, cells were treated with the various concentrations of each extract for 1 h. Dexamethasone (50 μM) was used as a positive control for the experiment. The following step was the stimulation with LPS (1 μg/mL) for 18 h. The effect of all the tested samples in the absence of LPS was also evaluated, in order to observe if they induced changes in Nitric oxide (NO) basal levels. In negative controls, no LPS was added. Both extracts and LPS were dissolved in supplemented DMEM (Taofiq, 2015).

2.5.2.2. Nitric oxide determination

For the determination of nitric oxide, a Griess Reagent System kit was used, which contains sulphanilamide, N-(1-napthyl)ethylenediamine hydrochloride (NED) and nitrite solutions. A reference curve of nitrite (sodium nitrite 100 µM to 1.6 µM; y=0.0066x+0.1349; R^2=0.9986) was prepared in a 96-well plate. The cell culture supernatant (100 µL) was transferred to the plate and mixed with sulphanilamide and NED solutions, 5-10 minutes each, at room temperature. The nitric oxide produced was determined by measuring the absorbance at 540 nm (microplate reader ELX800 Biotek), and by comparison with the standard calibration curve (**Figure 15**). The percentage of inhibition of the NO production was calculated, for each sample concentration, by the equation: [(Substrate concentration - Basal cells)/LPS – Basal cells] x100. The results were expressed as percent inhibition of NO production as compared to the negative control (100%) and EC_{50} values (concentration of sample that provides 50% inhibition of NO production) were also estimated (Taofiq, 2015).
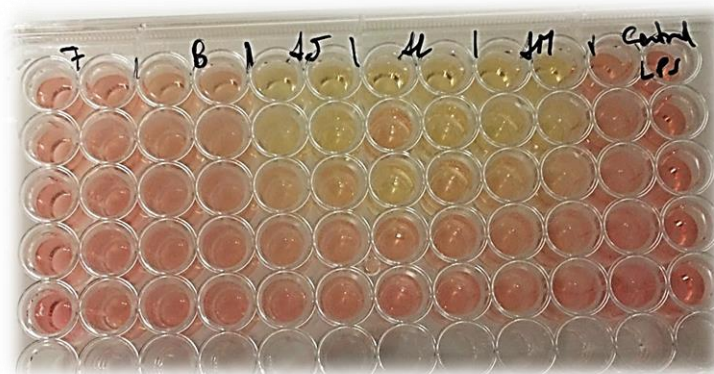
Figure 15: Microplate used in the evaluation of the NO assay.

2.5.3. Cytotoxic activity

For the cytotoxic activity assay, the methanolic extracts (section 2.3) were dissolved in water at a concentrated of 10 mg/mL. For the various assays, the extracts were then submitted to further dilutions from 10 mg/mL to 0.16 mg/mL. Ellipticine was used as positive control.

2.5.3.1. For human tumor cell lines

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

Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS and 2 mM glutamine (MCF-7, NCI-H460, HepG2 and HeLa cells) at 37 ºC, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7 and NCI-H460 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48 h with various extract concentrations. Following this incubation period, the adherent cells were fixed by adding cold 10% trichloroacetic acid (TCA, 100 μL) and incubated for 60 min at 4 ºC. Plates were then washed with deionised water and dried; sulforhodamine B solution (0.1% in 1% acetic acid, 100 μL, SRB) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried, the bound SRB was solubilised with 10 mM Tris (200 μL) and the absorbance was measured at 540 nm in the microplate reader mentioned above (**Figure 16**). The results were expressed as GI_{50} values (sample concentration that inhibited 50% of the net cell growth) in μg per mL (Barros *et al.,* 2013).

Figure 16: Microplate used in the evaluation of the cytotoxicity using human tumor cell lines.

2.5.3.2. For non-tumor cell lines

For evaluation of cytotoxicity in non-tumor cells, a cell culture from freshly harvested porcine liver and obtained from a local slaughterhouse was prepared according to the procedure described by Abreu and collaborators (2011), and referred by PLP2.

The explants were placed in culture dishes with DMEM supplemented with fetal bovine serum (FBS) (10%), 2 mM nonessential amino acids, 100 U/mL penicillin and 100 μg/mL

streptomycin, and placed in the incubator at 37 ºC with a humidified atmosphere containing 5% CO_2. Cultivation of the cells was continued with direct monitoring every 2-3 days using a phase contrast microscope (Icon Eclipse Ts 100). The cells were transferred to a 96 well plate at a density of $1x10^4$ cells/well and cultured in DMEM with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were treated for 48h with different concentrations and sample made up SRB assay (**Figure 17**) as described above. The ellipticine was used as a positive control. The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth).
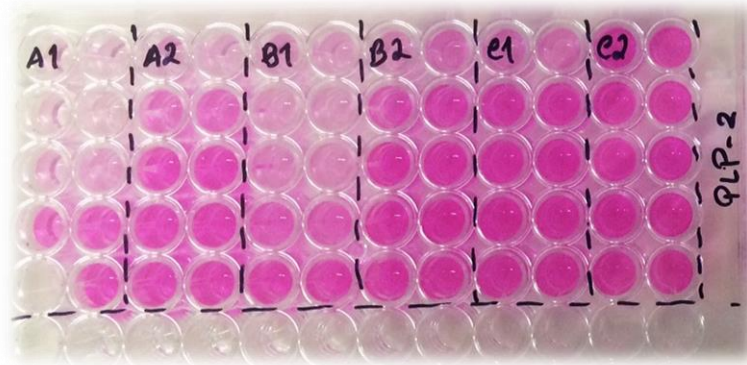
Figure 17: Microplate used in the evaluation of the cytotoxicity in non-tumor cell lines.

2.5.4. Statistical analysis

For each culture component, fruiting body and fungal species, three independent samples were analysed. Data were expressed as mean ± standard deviation. All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., Armonk, NY, 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 3.

3. RESULTS AND DISCUSSION

Mushrooms comprise a vast and yet largely untapped source of powerful new pharmaceutical products. In general, edible mushrooms are appreciated mainly for their nutritional value and most importantly for modern medicine. They represent an unlimited source of compounds with antioxidant, anti-inflamatory and cytotoxic potential, principally 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 in this study, the mycelia, as well as the culture media utilized in different stages of mushroom production, also presented a good source of valuable compounds. This study also evaluated the bioactivity of these mushrooms, such as antioxidant, anti-inflammatory and cytotoxic activity.

In antioxidant activity, non-enzymatic antioxidants such as β-carotene, ascorbic acid and α-tocopherol present in these mushrooms, may have an important role in the cellular response to oxidative stress, reducing reactive oxygen species (ROS), thus slowing the development of many chronic diseases as well as preventing lipid oxidation of food; these molecules have many applications as food additives, cosmetics, anti-aging and pharmaceutical products used in the treatment of chronic diseases related to oxidative stress (Fernandes, 2010).

For the anti-inflammatory activity, NO is a signalling molecule that plays critical role in the pathogenesis of inflammation. Although physiological levels of NO are required for events such as vasodilation, angiogenesis, and neurotransmission, the overproduction of NO under pathological conditions can be a toxic and pro-inflammatory mediator that induces inflammation. Therefore, the ability of these mushrooms to inhibit NO production under inflammatory stimuli is relevant to the development of anti-inflammatory agents (Taofiq, 2015).

We also evaluated the cytotoxic capacity of these mushrooms. Carcinogenesis is a process, which normally takes several years during which progressive genetic changes occur leading to malignant transformation. Cancer prevention is the best intervention in this process before invasive disease develops. Medicinal mushrooms have been used as

traditional medicine for the treatment and cancer prevention due to their important therapeutic properties (Ferreira *et al.,* 2010 and Younis *et al.,* 2014).

3.1. Mycelia prodution

In vitro production of mycelium can help to overcome difficulties associated with mushroom's availability related to their seasonality or adverse climatic conditions, being an important methodology for continuous production of mycelia with uniform characteristics, contaminants free and of high quality for food and farmaceutical purposes. The establishment of *P. ostreatus* and *A. bisporus in vitro* allowed the evaluation of differential growth rates and chemical characterizations in two media culture and two different physical conditions (solid vs. liquid).

After 7 days, the species *P. ostreatus* showed good growth on both media (PDA and MMNi solid medium). Mycelia were depigmented and cottony (**Figure 18 C and D**), and in PDA mycelia appeared as a thin layer (**Figure 18E**). The mycelia showed faster radial growth on PDA **(Figure 20)** and a higher biomass than on solid MMNi (fresh and dry weigth) (**Figure 22**). At the end of 3 weeks the entire plate was already covered with mycelia and the growth remained constant.

However, in liquid PDB and MMNi mycelia started to growth at day 8, and presented the same caracteristics than in solid media. In four weeks some of the mycelia already covered the PDB medium plate, but in liquid MMNi they stoped to grow and didn't cover the plate, presenting half of the diametre of PDB (**Figure 18 A and B**). In PDB liquid medium the *P. ostreatus* mycelia had a higher growth rate (**Figure 20**) and biomass than in liquid MMNi (fresh and dry weigth) (**Figure 21**).
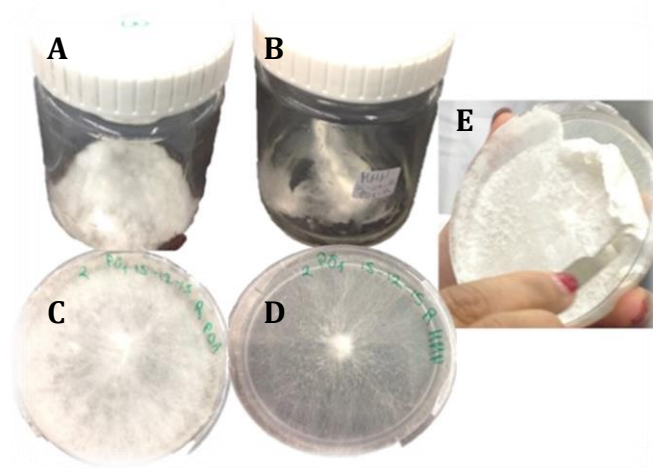
Figure 18: *Pleurotus ostreatus* mycelia cultivated *in vitro,* in both liquid (A-PDB and B- iMMN) and solid medium (C and E- PDA and D- iMMN).

A. bisporus mycelia showed little growth in all media (PDA, PDB, solid and liquid MMNi). This species presented depigmented and rhizomorphic mycelia (**Figure 19**).

This species started to grow in solid medium after 7 days. The mycelia grew in very small diameters and also grew into the culture medium, which hindered the removal of the mycelia; to solve this problem, mycelia were cultivated with cellophane paper (**Figure 19 J and K**). The radial growth was faster in MMNi than in PDA (**Figure 20**), despite the similarities in the produced biomass (fresh and dry weigth) (**Figure 21**). They stopped growing within 6 weeks and didn't cover the plate, (**Figure 19 H and I**).

In MMNi liquid medium they started to grow from day 14, but in PDB they started just 1 week after MMNi. The mycelium appeared as a grain of quinoa in result of agitation to stimulate growth (**Figure 19 F and G**). The radial growth was faster in MMNi (**Figure 20**) and biomass was also higher than in PDB (fresh weigth), although in dry weigth PDB showed higher biomass (**Figure 21**). They stopped growing within 8 weeks and didn't cover the plate. (**Figure 19**).

The main distinctive feature considering both radial growth patterns is that *P. ostreatus* exhibited a very fast initial growth in both media, although slower under liquid conditions reaching maximum radial growth after 14 days in solid medium while *A. bisporus* showed a slower and continuous growth rate along the 56 days period of culture, never reaching the complete top covering of the media.

Although radial growth was higher in solid than in liquid media for both species, biomass production was higher in liquid than in solid media being similar in both media

composition for *A. bisporus* but better in PDB for *P. ostreatus* and not very far from the biomass production in solid PDA medium.

Under the culture conditions here studied we can consider better growth results in PDA and PDB media for *P. ostreatus* with similar biomass production. *A. bisporus* exhibit similar biomass production in both media formulations but significantly higher under liquid conditions. After these growth results, chemical characterization of the samples will determine the best growth conditions for our main purposes.
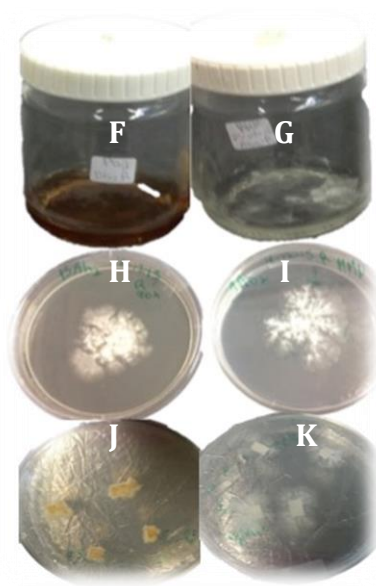
Figure 19: *Agaricus bisporus* mycelia cultivated *in vitro,* in both liquid (F- PDB and G- iMMN) and solid medium (H- PDA and I- iMMN).

A

B
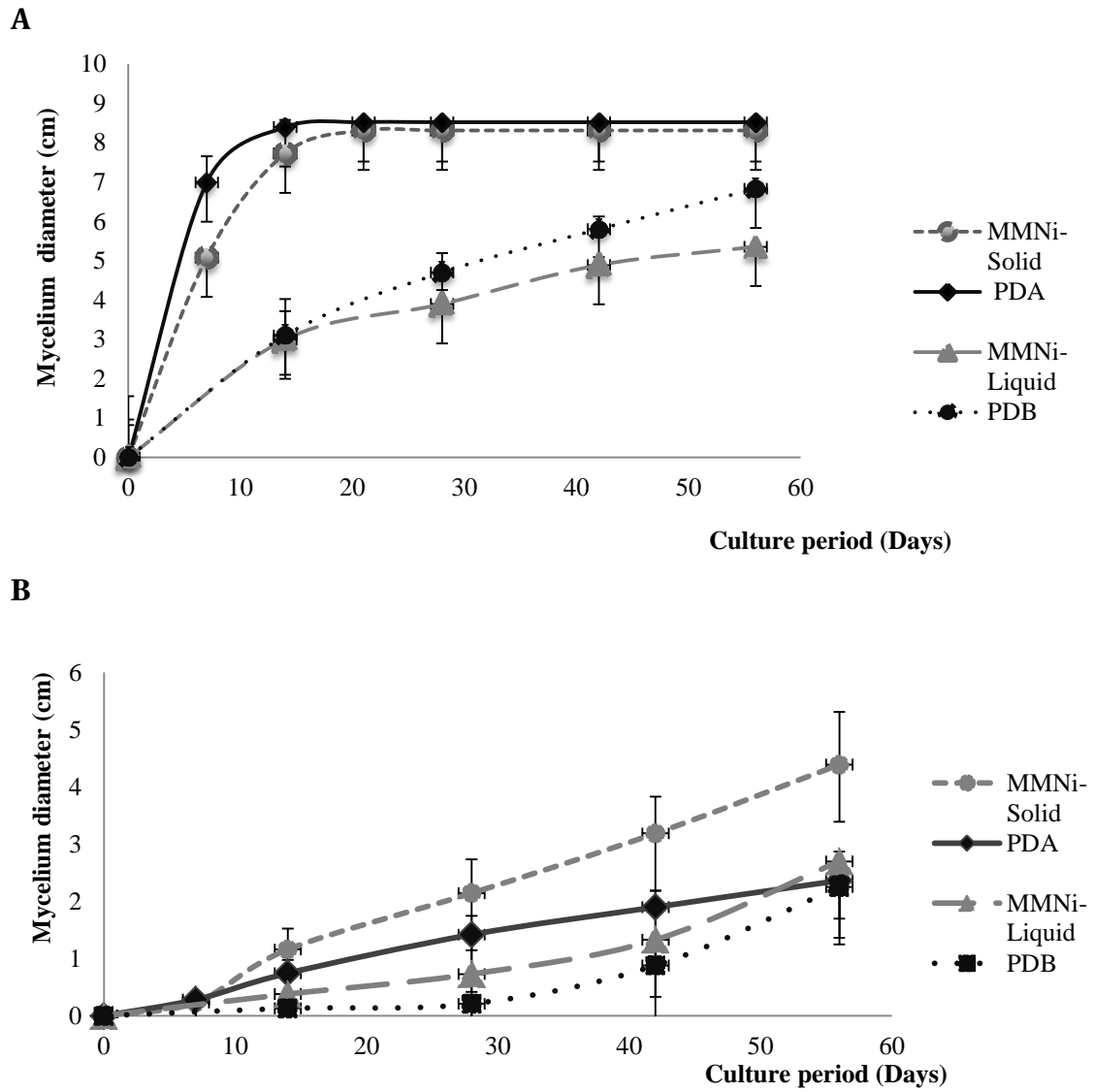
Figure 20: Radial growth of *A. bisporus* (A) and *P. ostreatus* (B) mycelia cultivated in different culture media throughout time.

A

Total biomass of *Agaricus bisporus* and *Pleurotus ostreatus* mycelia
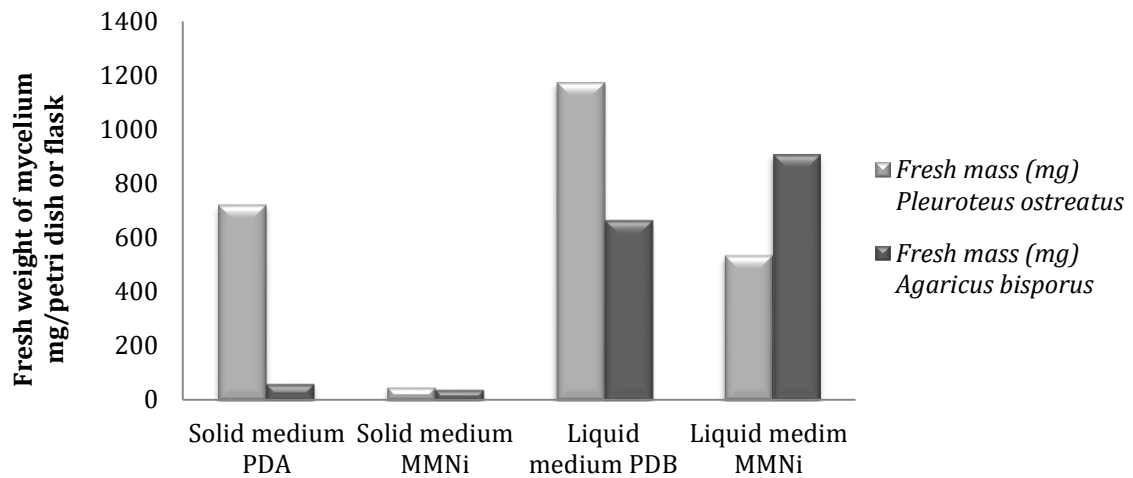
B
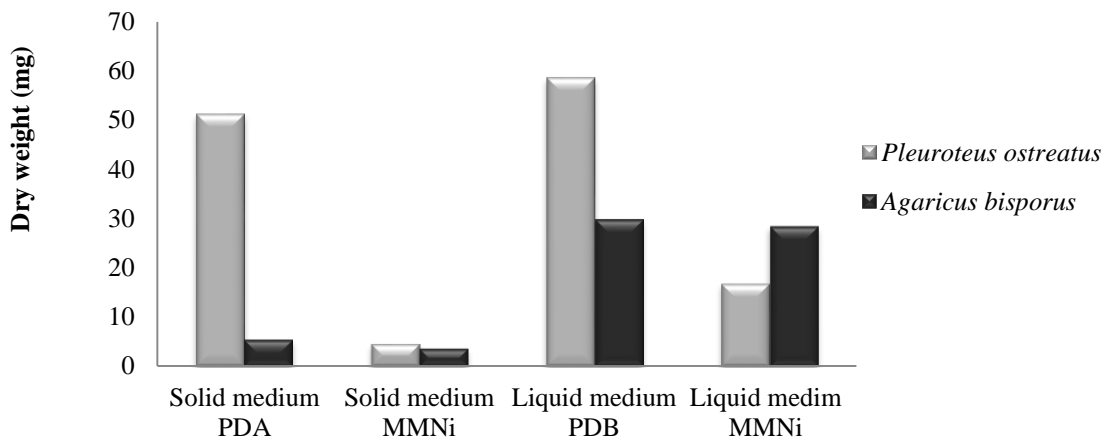
Figure 21: Total biomass of *A. bisporus* (A) and *P. ostreatus* (B) mycelia.

3.2. Chemical characterization of the extracts

Mushrooms have a lot of bioactive compounds including phenolic acids and ergosterol, also related to their nutraceutical potential (Barros *et al*., 2009 and Ferreira *et al*., 2009). Nutraceuticals present in mushrooms have been related to their antioxidant, anti-inflammatory and cytotoxic activities (Fernandes *et al.,* 2015). *A. bisporus* and *P.ostreatus* are sources of these compounds.

Mycelia, culture media (liquid culture media in which mycelia were dissolved) and fruiting bodies were compared in terms of bioactive compounds and properties.

Phenolic acids and ergosterol contents are presented in **Table 10,** while the chromatographic profiles of these compounds are shown in **Figure 22** and **23**. The highest content of ergosterol was found in the fruiting body of *A. bisporus* (17.4 ± 0.1 mg/g extract); it was not found in the culture medium. However, for *P. ostreatus* it was the mycelium cultivated in the PDB medium that showed higher quantity (18.56 ± 0.07 mg/g extract). The presence of ergosterol in these mushrooms was previously described by Villares and collaborators (2014). No ergosterol was also found in the PDB culture medium.

In general and comparing mycelia, *A. bisporus* gave lower content of ergosterol (ranging from iMMN solid - 2.87 ± 0.04 to PDB - 3.71 ± 0.04 mg/g extract) than *P. ostreatus* (ranging from iMMN liquid - 9.08 ± 0.03 to PDB - 18.56 ± 0.07 mg/g extract). But in fruiting bodies, it was *A. bisporus* (17.4 ± 0.1 mg/g extract) that showed higher content of ergosterol in comparison with *P. ostreatus* (9.7 ± 0.2 mg/g extract).

Among the phenolic acids, *p*-hydroxybenzoic and cinnamic acids were the major compounds in both mushrooms and in all samples. *p*-Hydroxybenzoic acid in mycelium grown in PDA reached quantities nearly five times higher in *A. bisporus* (1030 ± 59 µg/g extract) than in *P. ostreatus* (188 ± 34 µg/g extract). *P. ostreatus* fruiting body showed also high levels of this phenolic acid.

The phenolics compounds obtained in the present study are the same than those reported in literature for *A. bisporus* and *P. ostreatus* (unless the vanillic acid that could not be found in the present study) (Barros *et al.,* 2009). Cinnamic acid was found in higher amounts in *P. ostreatus* mycelium grown on PDB (438 ± 2 µg/g extract).

Regarding fruiting bodies, *A. bisporus* and *P. ostreatus* showed higher levels of *p*-hydroxybenzoic acid (129 ± 3 and 463 ± 11 µg/g extract, respectively) than of the other phenolic acids identified.

A. bisporus showed more quantity of cinnamic acid in the mycelium produced in PDA culture medium (134 ± 1mg/g extract), while *P. ostreatus* have higer concentration in the mycelium produced in PDB (438 ± 2 mg/g extract).

Protocatechuic acid was not found in *A. bisporus* like in previous studies (Reis *et al.,* 2012) and in *P. ostreatus* it was only found in the fruiting body (217 ± 16 mg/g extract) and in the mycelium produced in PDA (272 ± 7 mg/g extract). In previous studies protocatechuic

acid in *P. ostreatus* was found only in the fruiting body (Reis *et al.,* 2012 and Muszyńska *et al*., 2013).

A. bisporus gave higher levels of *p*-coumaric acid (10.4 ± 0.2 to 241 ± 9 mg/g extract) when compared with *P. ostreatus*. This acid was present in all samples of *A. bisporus*, unless in the culture media. But for *P. ostreatus*, it only appeared in the fruiting body (165 ± 2 mg/g extract) and in the mycelium produced in iMMN solid medium (17 ± 1 mg/g extract).

The differences observed between the studied samples and the values reported in literature are related with their different harvesting years and geographic locations that comprise diverse climatic conditions, which have influence on chemical compostion (Barros *et al.,* 2009; Reis *et al*., 2012 and Muszyńska *et al.,* 2013). For example, Reis *et al*. (2012) described the presence of another phenolic acid (gallic acid) in the same mushroom species but from cultivated origin (Reis *et al*., 2012).

The phenolic compounds and ergosterol contents (and not profiles) cannot be directly compared to previous studies because we expressed results in mg/g extract, and other literature expressed the resulst in dry weight basis (mg/g dw) (Barros *et al.,* 2009; Reis *et al*., 2012; Muszyńska *et al.,* 2013 and Barreira *et al.,* and Villares *et al.,* 2014).
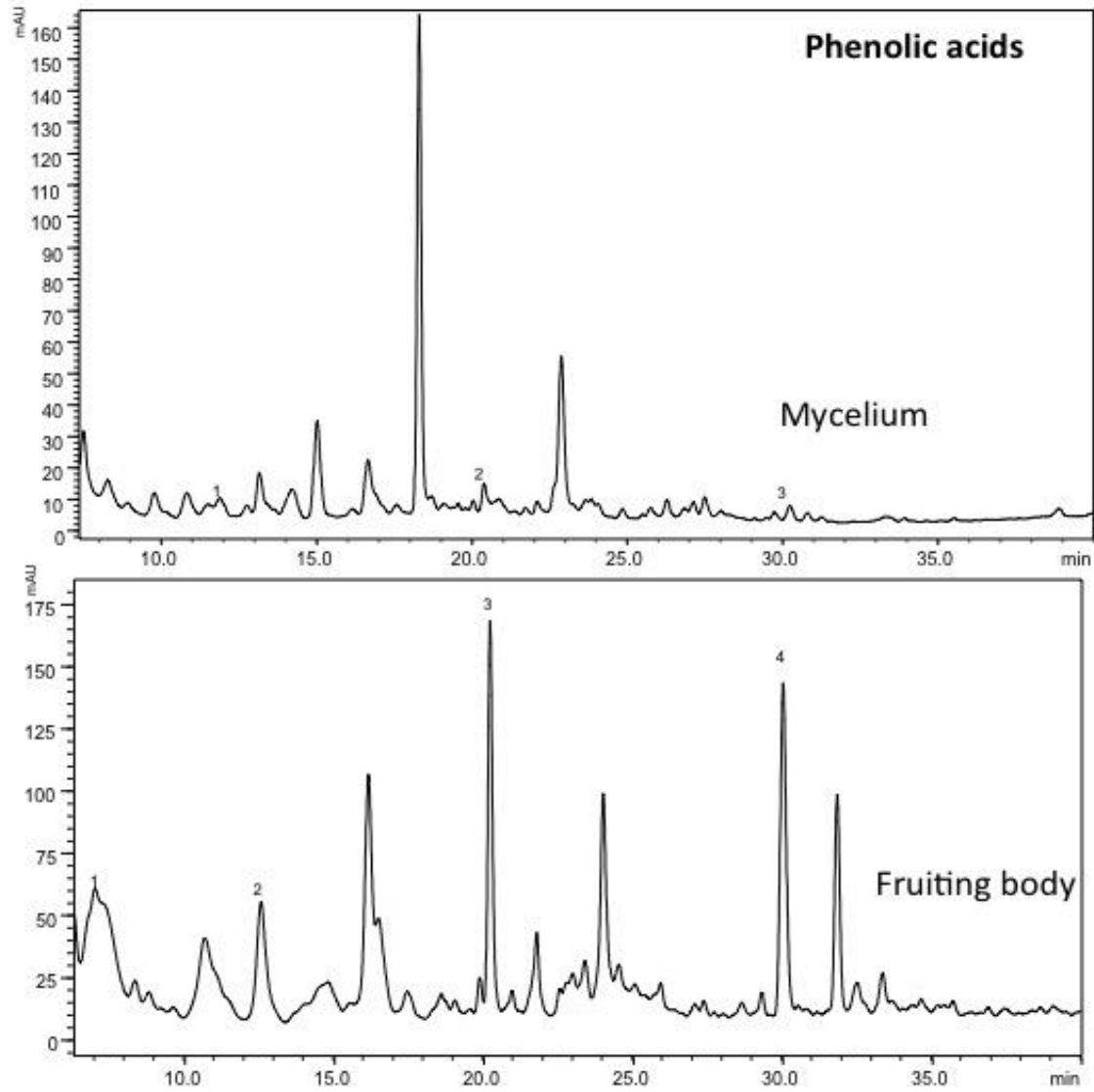
Figure 22: Chromatographic profiles of phenolic compounds of *A. bispous* (**Mycelium: 1-** *p*-hydroxybenzoic acid, **2-** *p*-coumaric acid, **3-** cinnamic acid) and *P. ostreatus* (**Fruiting body: 1-** protocatechuic acid, **2-** *p*-hydroxybenzoic acid, **3-** *p*-coumaric acid, **4-** cinnamic acid).
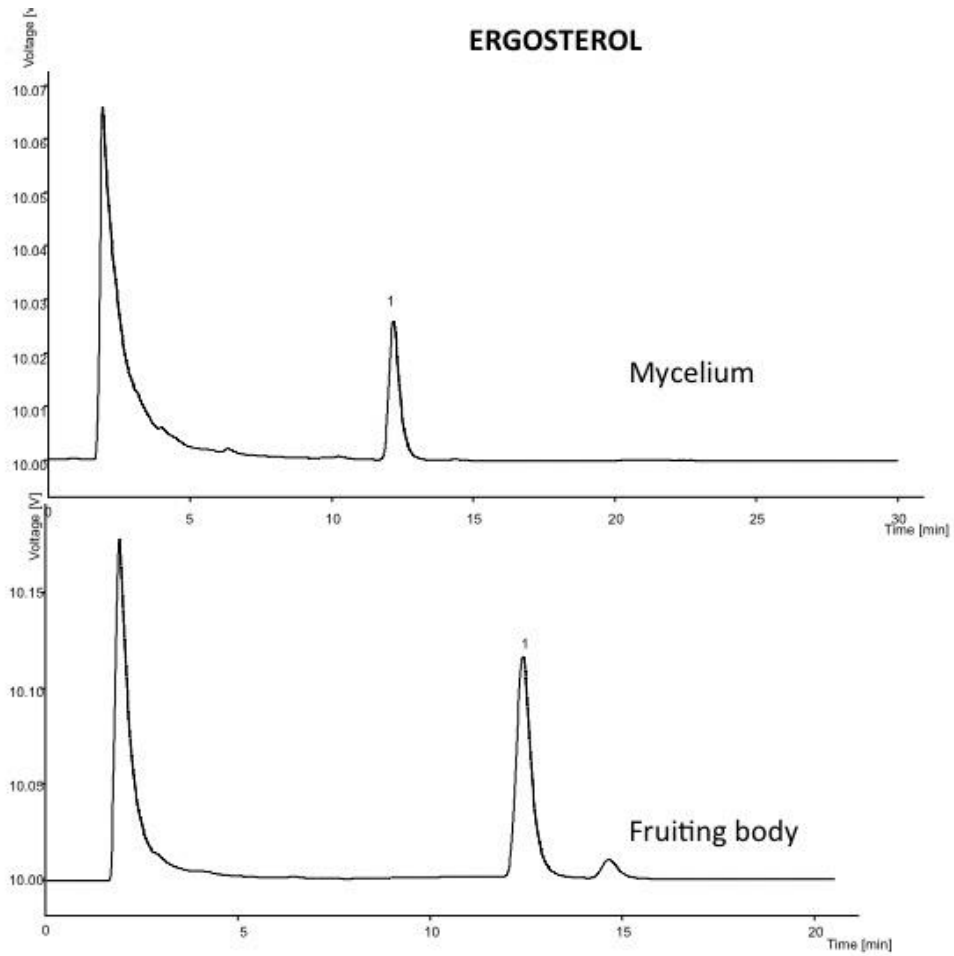
Figure 23: Chromatographic profiles of ergosterol *of A. bisporus* (Fruiting body **and** Mycelium: 1-**ergosterol).**

Table 10: Ergosterol (mg/g extract) and phenolic acids (µg/g extract) content in the mycelia and culture media of *A. bisporus* and *P. ostreatus.* The values corresponding to the fruiting body of both mushrooms (edible samples) are also presented. Values are given as mean±standard deviation.

		Ergosterol	Protocatechuic acid	*p*-Hydroxybenzoic acid	*p*-Coumaric acid	Cinnamic acid
		Agaricus bisporus				
Fruiting body (edible)		17.4±0.1	nd	129±3	17.9±0.7	15.3±0.1
iMMN liquid	Mycelium	3.27±0.05[b]	nd	12.5±0.1[d]	10.4±0.2[b]	9.0±0.1[c]
	Culture medium	Nd	nd	363±21[b]	Nd	14±3[c]
PDB	Mycelium	3.71±0.04[a]	nd	416±13[b]	50±1[b]	27±1[b]
	Culture medium	Nd	nd	56±2[c]	Nd	7.87±0.02[d]
iMMN solid	Mycelium	2.87±0.04[c]	nd	10±1[d]	24±1[c]	2.4±0.1[e]
PDA	Mycelium	3.69±0.01[a]	nd	1030±59[a]	241±9[a]	134±1[a]
p-values (n = 54)	Homoscedasticity[1]	0.143	-	0.052	0.029	0.072
	1-way ANOVA[2]	<0.001	-	<0.001	<0.001	<0.001
		Pleurotus ostreatus				
Fruiting body (edible)		9.7±0.2	217±16	463±11	165±2	142.8±0.4
iMMN liquid	Mycelium	9.08±0.03[d]	nd	38.2±0.5[d]	Nd	114.8±0.3[c]
	Culture medium	0.013±0.001[e]	nd	235±8[ab]	Nd	90±1[d]
PDB	Mycelium	18.56±0.07[a]	nd	266±4[a]	Nd	438±2[a]
	Culture medium	Nd	nd	125±3[c]	Nd	167±3[b]
iMMN solid	Mycelium	12.13±0.03[c]	nd	77.5±0.5[cd]	17±1	93±1[d]
PDA	Mycelium	16.0±0.3[b]	272±7	188±34[b]	Nd	75±5[e]
p-value (n = 54)	Homoscedasticity[1]	0.045	0.023	0.048	0.023	0.165
	1-way ANOVA[2]	<0.001	<0.001	<0.001	<0.001	<0.001

[1]Homoscedasticity 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 ($p<0.05$).

3.3. Antioxidant activity

The organisms are equipped against damage caused by free radicals with several defense mechanisms acting in different ways. As indicated, the antioxidants are natural molecules which prevent the uncontrolled formation of free radicals and ROS or inhibit their reaction with biological structures, interrupting the chain reaction and forming radicals having low reactivity which are easily removed from the body (Falcão, 2008 and Fernandes, 2010).

Maintaining balance between the production of free radicals and antioxidants cell defenses are an essential condition for the normal functioning of the body (Ferreira *et al.*, 2009). However, a balance between ROS and antioxidant defenses can be destabilized either by overproduction of ROS or the loss of the antioxidant defenses of cells. This imbalance is known as oxidative stress and, in this case, the excess ROS oxidize and can damage lipids, proteins and DNA of the cell, leading to alteration and inhibiting its normal function (Ferreira *et al.,* 2009). Ascorbic acid, *α*-tocopherol, *β*-carotene and other carotenoids, such as lycopene, and phenolic compounds are examples of antioxidants obtained from food. Mushrooms are a major source of these molecules (Fernandes, 2010).

The results obtained for each of the performed antioxidant activity assays, expressed as EC_{50} values, are shown in **Table 11. Figure 24** illustrates the test tubes or microplates in the preformed assays.
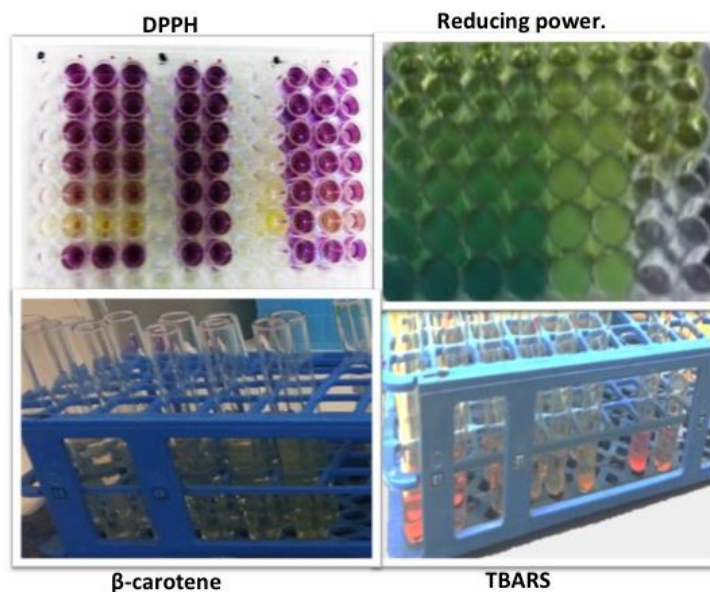
Figure 24: Test tubes and microplates used in the antioxidante activity assays.

Regarding fruiting bodies, the highest activity was obtained in the TBARS formation inhibition assay (*A. bispous*: 0.09±0.01 mg/mL; *P. ostreatus:* 0.090±0.002 mg/mL), followed by *β*-carotene bleaching inhibition (in the case of *P. ostreatus*: 0.7 ± 0.1 mg/mL) end reducing power (in the case of *A. bispous*: 0.81 ± 0.01 mg/mL). The lowest activity was observed in DPPH radicals scavenging activity assay (*P. ostreatus:* 3.2 ± 0.1 mg/mL; *A. bisporus*: 3.27 ± 0.03 mg/mL).

In general, the values obtained for *A. bisporus* (for example: Reducing power - 0.81 ± 0.01 mg/mL and TBARS - 0.09 ± 0.01 mg/mL) and *P. ostreatus* (for example: β-carotene - 0.7 ± 0.1 mg/mL and TBARS- 0.09 ± 0.002 mg/mL) fruiting bodies represent higher antioxidant activity than that reported previously (for example: *A. bisporus:* Reducing power - 1.80 ± 0.03 mg/mL and TBARS - 2.9 ± 0.5 mg/mL; *P. ostreatus*: *β*-carotene - 2.7 ± 0.2 mg/mL and TBARS - 2.6 ± 0.9 mg/mL), (except for DPPH in *A. bisporus*) (Reis *et al.,* 2012b). Also, for mycelia, the antioxidant activity of these species was higher (for example: *A. bisporus:* DPPH - 10.1 ± 0.03 mg/mL; *P. ostreatus:* DPPH - 17.1 ± 0.4 mg/mL) (except for *β*-carotene bleaching inhibition assay in *A. bisporus*) than that reported by those authors (for example: *A. bisporus:* DPPH - 40 ± 2 mg/mL; *P. ostreatus:* DPPH - 29 ± 6 mg/mL) (Reis *et al.,* 2012b).

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 *P. ostreatus*, the DPPH scavenging activity and *β*-carotene bleaching inhibition (3.2 ± 0.1 and 0.7 ± 0.1 mg/mL, respectively) was higher than those previously reported (5.11 ± 0.65 and 440 ± 43 mg/mL, respectively (Silva, 2015). *A. bisporus* also showed higher radical scavenging activity (3.27 ± 0.03 mg/mL) and reducing power (0.81±0.01 mg/mL) than a similar sample previously reported in literature (9.61 ± 0.07mg/mL and 3.63 ± 0.02 mg/mL, respectively) (Martins *et al*., 2012).

Considering the main purpose of this work, it was very interesting to discover that the antioxidant activity measured in the mycelia and in culture media was very close (in some cases better) to that verified in the fruiting bodies, emphasizing the high potential of these *in vitro* culture produced fungal 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's cultivation. Furthermore, the differences among the same culture media, after being used to grow each one of the mushroom species, indicate that the measured

antioxidant activity is in fact due to the mycelium grown in it, and not to the culture media components. Therefore, some compounds responsible for the antioxidant activity are released to the culture medium; while others are kept in the mycelium.

The culture media of *P. ostreatus* gave better results than the same components of *A. bisporus*. *P. ostreatus* in PDB culture medium showed higher *β*-carotene bleaching inhibition, TBARS formation inhibition and reducing power, while *A. bisporus* in MMN culture medium gave better results in DPPH scavenging activity and reducing power assays. Otherwise, *A. bisporus* mycelia produced in liquid medium MMN gave worst results than *P. ostreatus* in TBARS formation inhibition and *β*-carotene bleaching inhibition assays.

Table 11: Antioxidant activity (EC_{50} values, mg/mL) of the mycelia and culture media of *A. bisporus* and *P. ostreatus*. The values corresponding to the fruiting body of both mushrooms (edible samples) are also presented. Values are given as mean±standard deviation.

		DPPH scavenging activity	Reducing power	*β*-carotene bleaching inhibition	TBARS formation inhibition
		Agaricus bisporus			
Fruiting body (edible)		3.27±0.03	0.81±0.01	2.2±0.1	0.09±0.01
iMMN liquid	Mycelium	17.7.±0.3[b]	1.98±0.01[c]	4.4±0.2[ab]	0.7±0.1[b]
	Culture médium	18.2±0.5[b]	2.25±0.02[a]	4.7±0.1[ab]	1.4±0.1[a]
PDB	Mycelium	14.7±0.1[c]	1.23±0.01[e]	4.2±0.4[b]	0.19±0.01[cd]
	Culture médium	21.8±0.6[a]	2.11±0.02[b]	4.9±0.1[a]	0.36±0.02[c]
iMMN solid	Mycelium	13.31±0.1[d]	1.58±0.01[d]	4.5±0.1[ab]	0.22±0.003[cd]
PDA	Mycelium	10.1±0.03[e]	0.46±0.01[f]	4.2±0.1[b]	0.13±0.003[d]
p-value (n = 54)	Homoscedasticity[1]	0.005	0.174	0.045	0.002
	1-way ANOVA[2]	<0.001	<0.001	<0.001	<0.001
		Pleurotus ostreatus			
Fruiting body (edible)		3.2±0.1	1.79±0.01	0.7±0.1	0.09±0.002
iMMN liquid	Mycelium	56.9±0.6[a]	4.08±0.06[a]	4.6±0.2[a]	0.220±0.001[a]
	Culture médium	57.3±0.9[a]	3.66±0.01[b]	2.1±0.1[b]	0.22±0.01[a]
PDB	Mycelium	16.87±0.09[d]	1.85±0.01[c]	0.70±0.06[d]	0.04±0.0004[d]
	Culture médium	32.3±0.6[c]	1.9±0.1[c]	1.5±0.1[c]	0.17±0.01[b]
iMMN solid	Mycelium	52.4±1.4[b]	3.96±0.04[a]	2.4±0.1[b]	0.18±0.01[b]
PDA	Mycelium	17.1±0.4[d]	1.94±0.01[c]	0.92±0.01[d]	0.080±0.001[c]
p-value (n = 54)	Homoscedasticity[1]	0.059	0.108	0.016	0.003
	1-way ANOVA[2]	<0.001	<0.001	<0.001	<0.001
Trolox		41.68±0.28	41.43±1.27	18.21±1.12	22.84±0.74

[1]Homoscedasticity 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 ($p<0.05$).

3.4. Anti-inflammatory activity

The ability of both mushroom species to modulate the production of the inflammatory mediator NO (nitric oxide) was evaluated in RAW 264.7 macrophages by pre-treating cells with various concentrations of their methanolic extracts (**Table 12**).

LPS (lipopolysaccharide) is a cell wall component of Gram-negative bacteria, and plays a central role in the pathogenesis of several diseases (Taofiq, 2015). Macrophages are the main components of the innate immune system, and have essential regulation functions in several immunopathological conditions during the inflammatory process (Yoon *et al*., 2009).

When macrophages are exposed to LPS, the LPS binds to receptors that activate several signalling pathways that intern result in activation of NF-κB. When the NF-κB signalling pathway is activated, macrophages secrete NO and pro-inflammatory cytokines. Hence, natural agents that regulate the production of various cytokines and suppress the overproduction of NO may have protective roles in inflammation-related diseases such as cancer, diabetes and cardiovascular disease (Nam and Yuan *et al.,* 2006). Endotoxin LPS is able to induce the production of mediators like 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 and Taofiq, 2015).

Although phenolic acids were the compounds identified in the studied species, their content did not correlate with the inhibitory capacity of the extracts, which suggests that the contribution of these kinds of compounds to the anti-inflammatory activity is not relevant.

However, cinnamic acid derivatives, such as caffeic acid, have been previously described to down-regulate the production of proinflammatory cytokines in CACO-2 cells (Moro *et al*., 2012). By contrast, in other studies, cinnamic acid derivatives, such as caffeic acid, chlorogenic acid, ferulic acid and *p*-hydroxybenzoic acid, from an ethanolic plant extract, did not affect NO production in LPS-stimulated RAW 264.7 macrophages (Yu *et al*., 2009).

According to the obtained results none of the species showed anti-inflammatory activity (**Figura 25**). But, in previous studies presented by Moro *et al.* (2012) in methanolic extracts, *A. bisporus* showed an inhibition of NO production by 30% at 0.5 mg/mL and *P. ostreatus* 15% at 0.5 mg/mL. These authors explain that, pyrogallol that is only present in extracts of *A. bisporus*, *Cantharellus cibarius* Fr.*, Craterellus cornucopioides* (L.) Pers. and *Lactarius deliciosus* (L. ex Fr.) S.F.Gray cited by Palacios *et al.,* (2011), may be partly responsible for the anti-inflammatory activity. In the same way, pyrogallol from the medicinal plant *Emblica officinalis*, has been identified as one of the active compounds responsible for the anti-inflammatory effect of Emblica extracts in bronchial epithelial cells (Nicolis *et al.,* 2008). Synergisms between pyrogallol and other active compounds present in the extracts, such as flavonoids or cinnamic acid derivatives, could explain the differences in activity among the extracts studied by the mentioned authors.

According to the results reported by Taofiq (2015) for ethanolic extracts (EC_{50} values), *P. ostreatus* (96 ± 1 mg/mL) showed better results than *A. bisporus* (190 ± 6 mg/mL).

Figure 25: Microplates used in anti-inflammatory activity assay.

3.5. Cytotoxic activity

The cytotoxic activity of *A. bisporus* and *P. ostreatus* extracts against the four carcinoma cell lines (MCF-7, NCI-H460, HeLa and HepG2) and against a porcine liver primary cells culture (PLP2) is shown in **Table 12**.

As already stated, mushrooms are sources of several of powerful new pharmaceutical products. In particular, and most importantly for modern medicine, represent a powerful

source of compounds with potential antitumor and immunostimulating properties. Many of these compounds have been used in the treatment of many health problems, including cancer (Wasser, 2002).

Only the extracts prepared from *P. ostreatus* samples showed some activity against the four tumor cell lines (**Figure 26**). *A. bisporus* extracts could not inhibit these cell lines (up to the maximum assayed concentration: 400 μg/mL extract), ie, this species did not show any antitumor potential.
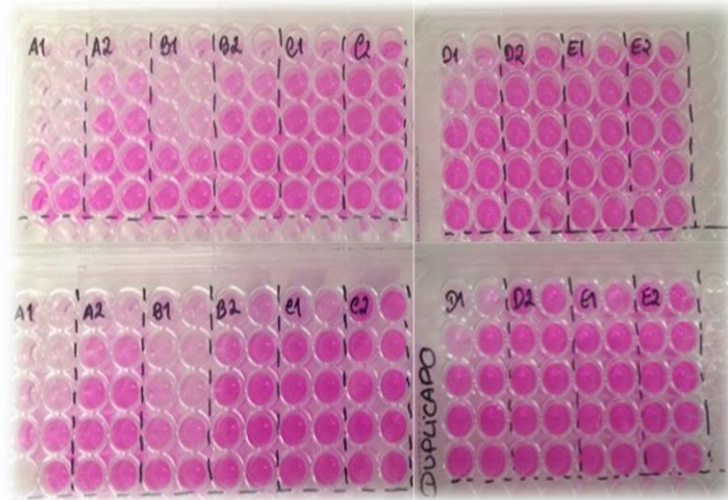
Figure 26: Microplate used in the evaluation of the cytotoxicity.

The extracts of *P. ostreatus* samples showed similar behavior in all cell lines (GI_{50} values varying from 224 ± 14 to 375.0 ± 0.1 μg/mL). In this species, the fruiting body showed activity only for NCI-H460 (GI_{50} = 306 ± 27 mg/mL). The mycelium produced in liquid medium MMN presented the highest cytotoxic effect against HepG2. All mycelia showed some activity in tumor cell lines, but none of the culture medium showed activity.

A previous work reported activities of *A. bisporus* and *P. ostreatus* mycelia (methanol extract) against HepG2 (54.9 ± 2.4 and 56.3 ± 2.5 mg/mL, respectively) and HeLa (38.7 ± 1.9 and 52.2 ± 2.5 mg/mL, respectively) (Younis *et al.,* 2014).

P. ostreatus samples that showed the highest cytotoxic activity gave also the highest content in phenolic acids and ergosterol.

The extracts from *A. bisporus* and *P. ostreatus* did not exhibit a toxic effect on the primary cell line. Interestingly, none of the assayed culture components inhibited 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.

Mushroom extracts can be used in combination with traditional chemotherapy or used as alternative sources for adjuvant cancer therapy, as some of these extracts have no adverse effects and activate the cells of the immune system. The bioactive compounds of mushrooms may complement classical cancer therapy and counter the side-effects of cancer, such as nausea, bone marrow suppression, anemia, and lowered resistance (Barros *et al.,* 2007 and Patel *et al.,* 2012).

Table 12: Cytotoxic (GI_{50} values. µg/mL) and anti-inflammatory activity (EC_{50} values. µg/mL) of the mycelia and culture media of *A. bisporus* and *P. ostreatus*. The values corresponding to the fruiting body of both mushrooms (edible samples) are also presented. Values are given as mean±standard deviation.

		MCF-7	NCI-H460	HeLa	HepG2	PLP2	RAW264.7
Agaricus bisporus							
Fruiting body (edible)		>400	>400	>400	>400	>400	>400
iMMN liquid	Mycelium	>400	>400	>400	>400	>400	>400
	Culture médium	>400	>400	>400	>400	>400	>400
PDB	Mycelium	>400	>400	>400	>400	>400	>400
	Culture médium	>400	>400	>400	>400	>400	>400
iMMN solid	Mycelium	>400	>400	>400	>400	>400	>400
PDA	Mycelium	>400	>400	>400	>400	>400	>400
p-value (n = 54)	Homoscedasticity	-	-	-	-	-	-
	1-way ANOVA	-	-	-	-	-	-
Pleurotus ostreatus							
Fruiting body (edible)		>400	306±27	>400	>400	>400	>400
iMMN liquid	Mycelium	239±18[b]	329±17[a]	286±23[b]	224±14[c]	>400	>400
	Culture médium	>400	>400	>400	>400	>400	>400
PDB	Mycelium	>400	>400	>400	375.0±0.1[a]	>400	>400
	Culture médium	>400	>400	>400	>400	>400	>400
iMMN solid	Mycelium	327±15[a]	>400[a]	>400	312±21[b]	>400	>400
PDA	Mycelium	330±28[a]	271±19[b]	338±1[a]	280±24[b]	>400	>400
p-value (n = 54)	Homoscedasticity	0.262	0.118	0.054	0.293	-	-
	1-way ANOVA	<0.001	<0.001	<0.001	<0.001	-	-
Dexamethaxone		-	-	-	-	-	15.70±1.1
Ellipticine		0.91±0.04	1.03±0.09	1.91±0.06	1.14±0.21	3.22±0.067	-

[1]Homoscedasticity among culture components was tested by the Levene test: homoscedasticity, p>0.05; heteroscedasticity, p<0.05. 2p<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).

CHAPTER 4.

4. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This study was designed to develop nutraceutical formulations based on mycelium of *Agaricus bisporus* and *Pleurotus ostreatus*, 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. ostreatus*, while incomplete Melin-Norkans medium (iMMN) solid media proved to be the best one for *A. bisporus*. Growth diameter rate is not correlated with the biomass of mycelium; for instance, radial growth was higher in solid than in liquid media for both species, but biomass production was higher in liquid than in solid media.

We obtained better growth results in PDA and PDB media for *P. ostreatus* with similar biomass production, and *A. bisporus* exhibited similar biomass production in both media formulations but significantly higher under liquid conditions.

For the chemical analysis, the 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 antitumor activities.

In general, *P. ostreatus* mycelia showed higher contents of ergosterol (9.08-18.56 mg/g extract). Cinnamic acid (75-438 μg/g extract) and *p*-hydroxybenzoic acid (463 μg/g extract) were also more abundant in the fruiting body of this species, but the mycelia of *A. bisporus* produced on PDA presented a higher amount of *p*-hydroxybenzoic acid (1030 μg/g extract).

Summing up, *A. bisporus* mycelia produced in PDA gave higher amounts of phenolic acids and ergosterol, but *P. ostreatus* had better contents of ergosterol, *p*-hydroxybenzoic acid and cinnamic acid in PDB and protocatechuic acid in PDA.

Likewise, the antioxidant activity was also higher among the *P. ostreatus* components, with the highest activity being obtained with the TBARS formation inhibition assay (EC_{50}

= 0.04 mg/mL). *A. bisporus*, showed better results of antioxidant properties in mycelia produced in PDA, and *P. ostreatus* had better results in PDB.

Extracts of *A. bisporus* did not show anti-inflammatory and cytotoxic activities up to the maximum assayed concentrations, contrarily to the observed for the mycelia of *P. ostreatus*, which showed some cytotoxicity, but did also not shown anti-inflammatory activity.

P. ostreatus mycelia showed cytotoxicity in tumor cell lines what did not happen with the fruiting body (except in NCI-H460, GI_{50} = 306 μg/mL). The highest activity was found against HepG2 (GI_{50} = 224 μg/mL). Therefore, it is difficult to assess which compounds are relevant for anti-inflammatory or antitumor activities of the studied sample. Furthermore, none of the species showed toxicity against non-tumor cells.

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

On the basis of obtaining unmodified natural products or nutraceutical formulations exhibiting antioxidant, anti-inflammatory and cytotoxic properties, to be used as food supplements or industrial applications that bring health benefits, the *in vitro* culture can be a great ally to obtain these bioactive compounds from fruiting body. That's why, science tools (genomic, proteomic and metabolomic) should be used to help further *in vitro* culture in order to obtain the most promising bioactive molecules to develop novel pharmaceutical products and nutraceuticals formulations with an overall positive impact on human health, but keeping environmental preservation.

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

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