Josiana Adelaide Vaz

STUDY OF ANTIOXIDANT, ANTIPROLIFERATIVE AND APOPTOSIS-INDUCING PROPERTIES OF WILD MUSHROOMS FROM THE NORTHEAST OF PORTUGAL.

ESTUDO DE PROPRIEDADES ANTIOXIDANTES, ANTIPROLIFERATIVAS E INDUTORAS DE APOPTOSE DE COGUMELOS SILVESTRES DO NORDESTE DE PORTUGAL.

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FACULDADE DE FARMÁCIA DA UNIVERSIDADE DO PORTO

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Josiana A. Vaz, Catarina Tavares, Gabriela M. Almeida, Anabela Martins, Isabel C.F.R. Ferreira, M. Helena Vasconcelos. Mushroom extract increases p53 expression and causes cell cycle arrest and apoptosis in a breast cancer cell line. **Annals of Oncology, 23, Suppl. 1, i28.** Targeted Anticancer Therapies 2012, 8-10 March 2012, Amsterdam, Holland. (Poster presentation).

Josiana A. Vaz, Gabriela M. Almeida, Anabela Martins, M. Helena Vasconcelos, Isabel C.F.R. Ferreira. *In vitro* growth inhibitory activity of the Portuguese wild mushroom *Clitocybe alexandri* in human tumour cell lines. **Revista Portuguesa de Farmácia, 2010, volume LII (nº4)**, 2º Encontro Nacional de Química Terapêutica, 28-30 November 2010, Coimbra. (Poster presentation).

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7. **Josiana A. Vaz,** Sandrina A. Heleno, Anabela Martins, Gabriela M. Almeida, M. Helena Vasconcelos, Isabel C.F.R. Ferreira. Bioactive properties of *Lepista inversa*. IV Jornadas de Análises Clínicas e Saúde Pública de Bragança, 9-10 April 2010, Bragança.

8. **Josiana A. Vaz**, Sandrina A. Heleno, Anabela Martins, Gabriela M. Almeida, M. Helena Vasconcelos, Isabel C.F.R. Ferreira. Antioxidant activity and growth inhibitory activity of Portuguese wild mushrooms, I3S Scientif Retreat (IBMC/INEB/IPATIMUP), 6 and 7 May 2010, Póvoa Varzim. P89.

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“aos que tanto quero e me querem...”

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ABSTRACT

Mushrooms are known as a powerful source of bioactive compounds including antioxidants, inhibitors of human tumour cell lines growth, inducers of apoptosis and enhancers of immunity. Indeed, many pre-clinical studies have been conducted in human tumour cell lines and in some cases a number of compounds isolated from mushrooms have followed to clinical trials. The Northeast of Portugal is one of the European regions with higher wild mushrooms diversity. However, to our knowledge, no studies had been conducted so far to verify their bioactivities.

The main aim of this work was the evaluation of the bioactive properties (antioxidant properties and growth inhibitory potential on human tumour cell lines) of wild edible mushrooms collected in the Northeast of Portugal.

Once properly identified, methanolic, ethanolic and boiling water extracts were prepared from thirty eight wild mushroom species collected in that region. Chemical characterization was obtained by high performance liquid chromatography (HPLC) coupled to a photodiode array detector (DAD) or to a refraction index detector (RI). Antioxidant activity assays were carried out in those extracts, including evaluation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals scavenging capacity, reducing power and inhibition of β-carotene bleaching. Extract-induced cell growth inhibition was assessed with the sulforhodamine B assay in four human tumour cell lines (NCI-H460 - lung cancer, MCF-7 -breast cancer, HCT-15 -colon cancer and AGS - gastric cancer). The effects on cell cycle profile and apoptosis were evaluated by flow cytometry and the effect on the expression levels of proteins related to cell cycle and apoptosis was further investigated by Western blotting.

Three wild edible mushroom species revealed growth inhibitory activity in the studied human tumour cell lines: *Clitocybe alexandri* ethanolic extract, *Lepista inversa* methanolic extract and *Suillus collinitus* methanolic extract. *C. alexandri* ethanolic extract induced an S-phase cell cycle arrest and increased the percentage of apoptotic cells, in the NCI-H460 cell line.

The analysed mushroom species also provided interesting antioxidant potential, mainly the boiling water extract of *L. inversa* which showed the highest DPPH radical scavenging activity, reducing power and β-carotene bleaching inhibition.

S. collinitus methanolic extract induced a slight increase in the number of cells in G1, with a concomitant decrease in the percentage of cells in the S phase of the cell cycle and an increase in the percentage of apoptotic cells, in the MCF-7 cell line. The combined use

of the *S. collinitus* methanolic extract and etoposide caused a greater decrease in the percentage of cell growth, when compared to either of them used individually, indicating the potential benefit of this combination.

The tested extracts were chemically characterized and protocatechuic, *p*-hydroxybenzoic, *p*-coumaric and cinnamic acids were the main compounds identified on the phenolic (methanolic and ethanolic) extracts, while mannitol, trehalose and arabinose were the main sugars found in the polysaccharidic (boiling water) extracts after hydrolysis.

The individual compounds identified in the extracts were submitted to a screening of tumour cells growth inhibitory activity, but only the phenolic acids and a related compound, cinnamic acid, presented activity. This compound was found to be the most potent one regarding cell growth inhibition in the NCI-H460 cell line.

The effect of the individual and combined treatment with the identified compounds was also evaluated. Cinnamic and protochatequic acids caused a statistically significantly reduction in the number of viable cells. In addition, *p*-hydroxybenzoic acid did not show any significantly reduction in the viable cell number. Nevertheless, it was verified that the concomitant use of the three compounds provided the strongest decrease in the viable cell number, suggesting a possible concomitant effect of those compounds.

Overall, the present work has contributed to further understand the bioactive potential of wild edible mushrooms from the Northeast of Portugal. This study allowed to identify some species with antioxidant or tumour cell growth inhibitory potential.

Keywords: Wild mushrooms, chemical characterization, antioxidant activity, antitumour activity, cell cycle, apoptosis.

RESUMO

Os cogumelos são conhecidos como uma poderosa fonte de compostos bioativos, incluindo antioxidantes, inibidores de crescimento de linhas celulares tumorais humanas, indutores de apoptose e imunoestimuladores. Já foram realizados vários estudos pré-clínicos em linhas celulares tumorais humanas e alguns compostos isolados de cogumelos estão em fase de ensaios clínicos.

O Nordeste de Portugal é uma das regiões Europeias com maior diversidade de cogumelos silvestres. No entanto, até agora não existiam estudos que verificassem as suas propriedades bioativas.

O objetivo principal deste trabalho foi a avaliação das propriedades bioativas (propriedades antioxidantes e potencial inibitório de crescimento de linhas celulares tumorais humanas) de cogumelos silvestres comestíveis colhidos no Nordeste de Portugal.

Uma vez devidamente identificados, prepararam-se extratos metanólicos, etanólicos e aquosos a partir de trinta e oito espécies de cogumelos silvestres colhidas nessa região. A caracterização química foi efetuada por cromatografia líquida de alta eficiência (HPLC) acoplada a um detetor de díodos (DAD) ou a um detetor de índice de refração (RI). Os ensaios de atividade antioxidantes, realizados nestes extratos, incluíam a avaliação da atividade captadora de radicais 2,2-difenil-1-picril-hidrazilo (DPPH), poder redutor e inibição da descoloração do β-caroteno. A inibição do crescimento celular foi avaliada com o ensaio Sulforrodamina B em quatro linhas celulares tumorais humanas (NCI-H460 - pulmão, MCF-7- mama, HCT-15 - cólon e AGS - gástrico). Os efeitos sobre o perfil do ciclo celular e apoptose foram avaliados por citometria de fluxo e o efeito sobre os níveis de expressão de proteínas relacionadas com ciclo celular e apoptose celular, foi investigado por Western blotting.

Três espécies de cogumelos silvestres comestíveis revelaram atividade inibitória do crescimento de linhas celulares tumourais humanas. O extrato etanólico de *Clitocybe alexandri*, o extrato metanólico de *Lepista inversa* e o extrato metanólico de *Suillus collinitus* revelaram ser os mais potentes.

O extrato etanólico de *C. alexandri* induziu uma paragem do ciclo celular em fase S e promoveu um aumento da percentagem de células apoptóticas, na linha celular tumoural humana de pulmão testada.

As espécies de cogumelos analisadas também revelaram um interessante potencial antioxidante, principalmente o extrato aquoso de *L. inversa* que apresentou a maior

atividade captadora de radicais DPPH, poder redutor e inibição da descoloração do β-caroteno.

O extrato metanólico de *S. collinitus* induziu um ligeiro aumento no número de células em G1, com uma concomitante diminuição na percentagem de células na fase S do ciclo celular e um aumento na percentagem de células apoptóticas na linha celular tumoural humana de mama testada. O uso combinado do extrato metanólico de *S. collinitus* e de etoposideo causou uma maior diminuição na percentagem de crescimento celular, quando comparado com qualquer um deles usados individualmente, indicando o potencial benefício desta combinação.

Os extratos testados foram caracterizados quimicamente, tendo sido os ácidos protocatéquico, *p*-hidroxibenzóico, *p*-cumárico e cinâmico os principais compostos identificados nos extratos fenólicos (metanólico e etanólico), enquanto o manitol, a trealose e a arabinose foram os principais açúcares encontrados no extrato polissacarídico (aquoso), após hidrólise.

Os compostos individuais identificados nos extractos foram submetidos a uma avaliação da atividade inibitória de crescimento celular, mas apenas os ácidos fenólicos e um composto relacionado, o ácido cinâmico, apresentaram atividade. Este composto foi o mais potente em relação ao efeito de inibição de crescimento celular, na linha celular tumoural humana NCI-H460.

O efeito do tratamento individual e combinado dos compostos identificados também foi avaliado. Os ácidos cinâmico e protocatéquico causaram uma redução estatisticamente significativa no número de células viáveis. Por sua vez, o ácido *p*-hidroxibenzóico não demonstrou qualquer efeito sobre as células testadas. No entanto, verificou-se que a utilização simultânea dos três compostos promoveu uma diminuição substancial no número de células viáveis, sugerindo um possível efeito concomitante dos referidos compostos.

Concluindo, o presente trabalho contribuiu para uma melhor compreensão do potencial bioativo de cogumelos silvestres comestíveis do Nordeste de Portugal. Este estudo permitiu identificar algumas espécies com propriedades antioxidantes ou inibitórias do crescimento de células tumourais.

Palavras- chave: Cogumelos silvestres; caracterização química; atividade antioxidante; atividade antitumoural; ciclo celular; apoptose.

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ABBREVIATIONS LIST

ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
CAT	Catalase
CDKs	Cyclin-dependent kinases
DISC	Trimerized receptor-ligand complex
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
dw	Dry weight
FADD	Fas-associated death domain protein
FAO/WHO	Food and Agriculture Organization of United Nations/ World Health Organization
GC-MS	Gas chromatography coupled to mass spectrometry
GPH-Px	Glutathione peroxidase
GSH	Glutathione
Gred	Glutathione redutase
HIV	Human immunodeficiency virus
HPLC-DAD/ESI-MS	High-performance liquid chromatography coupled to photodiode array detector/electrospray ionization mass spectrometry
HPLC-UV	High-performance liquid chromatography coupled to UV detector
ILSI	International Life Sciences Institute Europe
INK4a/ARF	Inhibitor of kinase 4/alternative reading frame
LDL	Low-density lipoproteins
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCI	National Cancer Institute
Px-GPH	Glutathione peroxidase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SRB	Sulphorhodamine B
TBARS	Thiobarbituric reactive substances
TNF	Tumour necrosis factor
UV	Ultraviolet radiation
XTT	2,3-Bis-(2-methoxy-4- nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

CHAPTER I.

INTRODUCTION

1. FUNGI

Fungi are nowadays known scientifically as an individual group of organisms, but had for long been classified as plants. It was only in 1969 that Whittaker reclassified these organisms into a separate kingdom, the Fungi. Fungi were then divided in two groups, according to their size, the Micromycetes, for microscopic species and the Macromycetes or Macrofungi, which included all those which are avidly collected by enthusiasts in the woods and fields (Whittaker, 1969). Mushroom is "a macrofungus with a distinctive fruiting body, which can be hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand". In fact, the fruiting body of the fungus is the structure, which is called mushroom, while mycelium is the vegetative part comprising a system of branching threads and cordlike stands, which could produce the fruiting body under favourable conditions (Chang, 2008).

Although mycology has been classically considered a branch of botany, there is evidence that the kingdom Fungi is more closely related to Animalia than to Plantae. Both animals and fungi have chitinous structures, storage of glycogen, and mitochondrial codon UGA encoding tryptophan, among other phylogenetic features (Nikoh *et al.*, 1994).

True Fungi (Mycota or Eumycota) have general characteristics that justify their classification as a separate kingdom: they are all eukaryotic, most are filamentous with individual cells constituting filaments called hyphae. Hyphae grow apically and branch to form a network referred to as mycelium. Some, like yeast, are unicellular. Fungi cells are surrounded by a cell wall, containing chitin and glucans and their nuclei are mainly haploid with cells often multinucleate. Reproduction can be both sexual and assexual resulting in spore production. From the metabolic point of view, they are all achlorophyllous and though incapable of photosynthesis being chemoheterotrophic (chemo-organotrophic). They possess a characteristic range of storage compounds like trehalose, glycogen, sugar alcohols and lipids. In terms of ecology, they can be free-living organims or may form intimate relationships with other organisms (plant roots_-mycorrhizae, algae - lychen, other fungi) and they can establish all types of trophic relations, being saprobiotic, parasitic or symbiotic (Hibbet, 2007).

Prior classification systems of Fungi based on morphology were updated to more accurately reflect phylogenetic relationships determined by molecular tools (Hibbet,

2007). Fungi with non-septate or irregularly septate hyphae and thick-walled spores were traditionally placed in the phylum Zygomycota. However, evidence for a monophyletic Zygomycota is lacking (Seif *et al.*, 2005), and Hibbett (2007) proposed the separation of the Zygomycota into four unordered subphyla (Entomophthoromycotina, Kickxellomycotina, Mucoromycotina, Zoopagomycotina). The separation of the arbuscular mycorrhizal fungi (that lack septa in hyphae but also lack zygospores) into the phylum Glomeromycota has been proposed by Schüßler *et al.* (2001).

The morphological characters shared between Basidiomycota and Ascomycota such as regularly septate hyphae and a dikaryotic stage (two separate and different nuclei in a single hyphal segment) in the life cycle usually has been interpreted as support for a close relationship between both taxa. Numerous phylogenetic studies such as SSU rDNA (Berbee and Taylor, 1992), RNA polymerase genes (Liu *et al.*, 2006), and mitochondrial genome sequencing (Seif *et al.*, 2005) provide strong support for this relationship. A subkingdom desiganted Dikarya is proposed (Hibbett, 2007), creating a division between the two well defined phyla (Basidiomycota and Ascomycota) and the remaining early diverging lineages whose relationships are not precisely known.

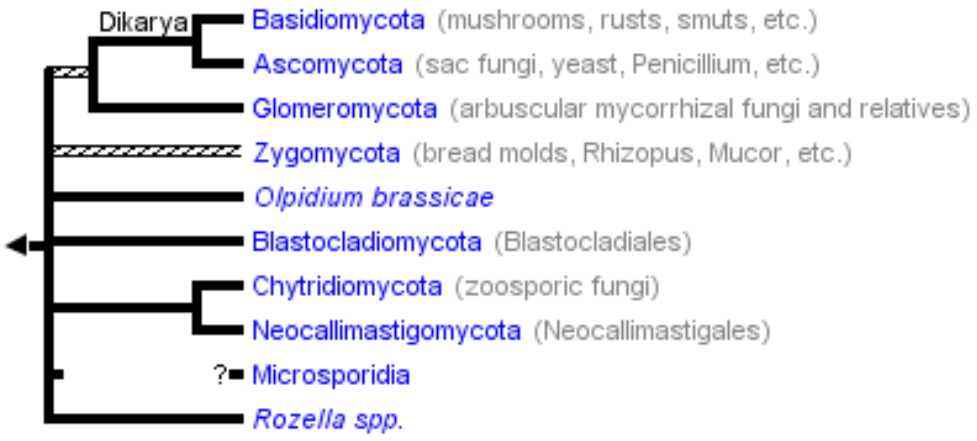


Figure 1. Phylogeny of Kingdom Fungi according to Hibbett et al., 2007 [adapted from (Balckwell *et al.* 2012).

Basidiomycota is one of two large phyla that, together with the Ascomycota, comprise the subkingdom Dikarya (often referred to as the "higher fungi") within the

kingdom Fungi. The number of mushroom species on earth is estimated to be 140,000, but only 10% are already known. Assuming that the proportion of useful mushrooms among the undiscovered and unexamined mushrooms will be only 5% (an estimated small % which seems logical), this implies that there may exist 7,000 yet undiscovered species of possible benefit to mankind (Hawksworth, 2001).

The Basidiomycota contains about 30,000 described species, which is 37% of the described species of true Fungi (Kirk *et al.* 2001). The most conspicuous and familiar Basidiomycota are those that produce mushrooms, which are sexual reproductive structures. The Basidiomycota also includes yeasts, single-celled forms (Fell *et al.* 2001) and asexual species. Basidiomycota are found in virtually all terrestrial ecosystems, as well as freshwater and marine habitats (Kohlmeyer and Kohlmeyer, 1979; Hibbett and Binder, 2001). They are unicellular or multicellular, sexual or asexual, and terrestrial or aquatic and so variable that it is impossible to identify any morphological characteristics that are both unique to the group and constant in the group. Their most diagnostic feature is the production of basidia, which are the cells on which sexual spores are produced, and from which the group takes its name. A long-lived dikaryon, in which each cell in the thallus contains two haploid nuclei resulting from a mating event, is another characteristic feature. Finally, clamp connections are a kind of hyphal outgrowth that is unique to Basidiomycota, although they are not present in all of them (Swann and Hibbett, 2007).

The Agaricomycotina is one of three major clades of Basidiomycota (with Pucciniomycotina and Ustilaginomycotina). The Agaricomycotina contains about 20,000 species already described, which is almost 70% of the know Basidiomycota (Hibbett, 2007). About 98% of the species of the Agaricomycotina are in the clade Agaricomycetes, which includes mushrooms, bracket fungi, puffballs, and others. Tremellomycetes and Dacrymycetes are the other major groups (Hibbett, 2007). Agaricomycetes contains approximately 16,000 described species, which is 98% of the described species in the Agaricomycotina (Kirk *et al.*, 2001). Agaricomycetes produce mushrooms, and are therefore the most familiar and conspicuous of all Fungi. Other Fungi also produce macroscopic fruiting bodies, but the diversity of forms in the Agaricomycetes is unmatched (Hibbett, 2007).

Fruiting bodies of Agaricomycetes range from millimeter-scale, to the giant polypores (Burdsall *et al.*, 1996). Agaricomycetes include not only the largest fruiting bodies in Fungi, but perhaps the largest and oldest individuals in any group of

organisms. Agaricomycetes act as decayers, pathogens, parasites, and mutualistic symbionts of both plants and animals. They make their broadest ecological impacts through their activities as wood-decayers and ectomycorrhizal symbionts of forest trees (such as pines, oaks, dipterocarps, and eucalypts; Rayner and Boddy, 1988; Smith and Read, 1997). Agaricomycetes are widespread in virtually all terrestrial ecosystems (Hibbett, 2007).

Agaricomycetes include the majority of the edible mushrooms (although truffles and morels are Ascomycota). Cultivated edible Agaricomycetes are decayers that have been domesticated, such as button mushrooms (*Agaricus bisporus*), shiitake (*Lentinula edodes*), oyster mushrooms (*Pleurotus ostreatus*), and others. Most of the wild-collected edible species are mycorrhizal and (this makes them difficult or quite impossible to cultivate) such as porcini (*Boletus edulis*), chanterelles (*Cantharellus cibarius*), and matsutake (*Tricholoma matsutake*). Some species of Agaricomycetes produce secondary metabolites that make them toxic or hallucinogenic (or bioluminescent) (Hibbett, 2007). Furthermore, approximately 700 species of higher Agaricomycetes have been already found to possess significant pharmacological activities (Mizuno, 1995; Wasser, 2002).

Since ancient times many mankind cultures have used mushrooms as both food and medicine. Nowadays, the fruiting body of edible mushrooms is usually the material which is collected and consumed as food but several types of products, from fruiting bodies to mycelia, are commercialized as dietary supplements given their potential therapeutic effects, and/or consumed in the form of capsules, tablets or extracts (Chang and Buswell, 2003). Recently, these fungi have been considered as having a potential market as functional foods. At the same time, some species are greatly appreciated for their extremely high value in gourmet cooking (Chang, 2008).

1.1 NUTRITIONAL PROPERTIES

Edible mushrooms are widely consumed in many countries as food. Given their attractive taste, aroma and nutritional values, edible mushrooms are valuable components of the diet. Their culinary and commercial value is mainly due to their organoleptic properties such as their texture and flavour, being possible to distinguish edible mushroom species on the basis of their characteristic odor or aroma (De Pinho *et al.*, 2008; Kalac, 2009; Zawirska-wojtasiak *et al.*, 2009).

Edible mushrooms are essentially constituted by water (81.8 to 94.8%). This variability is related to the mushroom species and to the harvest, growth, cooking and storage conditions. Due to the high moisture content, fresh edible mushrooms have a short shelf life, therefore the consumption of wild edible mushrooms throughout the year is not possible unless an appropriate storage processing is performed (Kalac, 2009; Guillamón *et al.*, 2010).

Their important nutritional value has been attributed to high protein, fiber, vitamin, mineral, carbohydrates and water contents and to low-fat levels.

In general, mushrooms are quite rich in protein, having an important content of essential amino acids (Mattilda *et al.*, 2001). The amino acids composition of mushroom proteins are comparable to animal proteins (Flegg and Maw, 1997; Gruen and Wong, 1982; Kalac, 2009).

For example, the protein content found in mushrooms may vary from 15.2 g/100 g dry weight (dw) in *Lentinus edodes* to 80.93 g/100 g dw in *Agaricus bisporus* (Manzi *et al.*, 1999). The levels of essential amino acids in mushrooms have been reported to vary widely among species (Manzi *et al.*, 1999; Guillamón *et al.*, 2010). According to FAO/WHO, they are considered rich in glutamic acid, aspartic acid and arginine. However, their proteins are deficient in methionine and cysteine (Manzi *et al.*, 1999). It is believed that the consumption of mushrooms will have a tendency to increase in coming years, precisely because they are an important source of proteins and amino acids that might replace animal proteins, thereby reducing the risks associated with its use in the human diet (Kalac, 2009).

Mushrooms are also a rich source of numerous dietary fibers. In general, a remarkably high or appreciable level of total fiber ranging from 5.5 to 42.6 g/100g dw was obtained from the Boletus group, *Agrocybe aegerita*, *Agaricus bisporus*, *Pleurotus eryngii* and *ostreatus*, in which β-glucans are the major fiber polysaccharides together with chitin (Manzi *et al.*, 2001; Manzi *et al.*, 2004). Dietary fiber in mushrooms shows higher levels of insoluble dietary fiber (2.28–8.99 g/100 g edible weight) than soluble dietary fiber (0.32–2.20 g/100 g edible weight) (Manzi *et al.*, 2000; Manzi *et al.*, 2004). The β-glucans represent from 4 to 13% of the total dietary fiber, depending on mushrooms species (Kalac, 2009).

The range of reported concentrations of carbohydrates varies from 35 to 70% dw. Nonetheless, despite the different profile of sugars among species, mannitol and trehalose are the main sugars in many analyzed mushrooms. For example in *Lactarius deliciosus*, *Chantarellus cibarius*, *Agaricus bisporus* and *Volvariella volvacea*, mannitol was the most abundant sugar, while trehalose predominates in *Pleurotus ostreatus*, *Boletus edulis*, *Lepista nuda* and *Calocybe gambosa*. Other sugars only occur either in small amounts or remain undetected (Bano and Rajarathnam, 1988; Longvah and Deosthale, 1998; Díez and Alvarez, 2001; Manzi *et al.*, 2004; Barros *et al.*, 2007; Barros *et al.*, 2008b).

Furthermore, these fungi have a nutritionally significative content of vitamins (B1, B2, B12, C and D) and mineral elements (Ca, K, Mg, Na, P, Cu, Fe, Mn and Se). Indeed, mushrooms are a good source of vitamins, based on the high levels of riboflavin (vitamin B2), niacin and folates and traces of vitamin C, vitamin B1, vitamin D, β-carotene (precursor of vitamin A), vitamin E and vitamin B12 (Mattilda *et al.*, 2001; Heleno *et al.* 2010). Mushrooms appear as the only non-animal-based food source containing vitamin D, and hence they are the only natural vitamin D source for vegetarians. Indeed, vitamin D2 content is considerable in a number of wild mushroom species, but unfortunately is almost absent in cultivated species (Mattila *et al.*, 1994; Mattilda *et al.*, 2001).

As compared with vegetables, mushrooms proved to provide a reasonable content of many mineral elements (6–10.5% dw). The main constituents in the ash are potassium and, depending on the mushroom, phosphorus or magnesium, in addition to calcium, copper, iron and zinc (Mattilda *et al.* 2001).

Dietary mushrooms provide low amounts of fat. Generally, unsaturated fatty acids are predominant over saturated fatty acids especially palmitic acid (C16:0), oleic acid (C18:1) and linoleic acid (C18:2) (Longvah and Deosthale, 1998; Barros *et al.*, 2007). The remaining fatty acids are only found in small amounts, except in the case of the *Lactarius* species which contains an abundant amount of stearic acid (C18:0) (Barros *et al.*, 2007; Heleno *et al.*, 2009). Linolenic acid (C18:3) is the precursor for 1-octen-3-ol (also known as mushroom alcohol), the principal aromatic compound in most fungi, which characteristically and distinctively contributes to mushrooms flavour (Maga, 1981).

With no doubt, edible mushrooms in fresh, cooked or processed forms are a nutritionally sound and tasteful food source for most people and can be a significant dietary component for vegetarians (Breene, 1990).

1.2 MEDICINAL PROPERTIES

In addition to being nutrient-rich foods and quite tasty, edible mushrooms have been known to be a source of bioactive products (Hobbs, 1995).

In this context, edible mushrooms can be considered as a functional food. The International Life Sciences Institute (ILSI Europe), states that "a food can be considered as "functional" if it is satisfactorily shown to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to both a better state of health and welfare and/or reduction of disease risk" (Thomas and Earl, 1994; Diplock *et al.*, 1999). However, food as medicine reinforces the paradigm of functional foods. Functional foods cannot claim to cure diseases but, increasingly, evidence is being produced that supports the role of some functional foods in disease prevention (Jones and Jew, 2007; Sirò *et al.*, 2008).

Wild mushrooms can be found in old books of traditional medicine, especially in the Orient, as specific pharmacological agents (Hobbs, 1995). Almost all important medicinal mushrooms are under a large-scale artificial cultivation (Smith, 1972). According to the definition of functional food by the International Life Sciences Institute in Europe (Diplock *et al.*, 1999), the mushroom is now gaining worldwide recognition as a functional food concerning improvement, prevention or treatment of some diseases (Mattilda *et al.*, 2000; Cheung, 2008).

Functional food science is now considered as a part of nutritional science, in which the primary objectives are to maintain good health, improve homeostasis and to create the conditions for disease risk reduction. In this way it could be seen to be quite distinct from the medical and pharmaceutical sciences, where the objectives are mainly to cure or control diseases (Diplock *et al.*, 1998; Saris, 1998).

Several studies have been published in which different species of fungi have shown potential in the prevention and treatment of a number of human diseases (Wasser and Weis, 1999a; Chang, 2008; Ferreira *et al.*, 2009; Ferreira *et al.*, 2010; Guillamón *et al.*, 2010; Xu *et al.*, 2011).

In Asian countries, like China and Japan, mushrooms such as Lingzhi (*Ganoderma lucidum*), Shiitake (*Lentinus edodes*), and Yiner (*Tremella fuciformis*) that have been collected, cultivated and used for hundreds of years, are being evaluated as edible and

medicinal resources. Indeed, most traditional knowledge about the mushrooms as food and medicinal agents comes from these species (Hobbs, 1995; Miles and Chang, 1997; Wasser, 2002).

Several genus of mushrooms are known to have various therapeutic properties, for example genus species like *Lentinus* (Lentinula), *Auricularia*, *Hericium*, *Grifola*, *Flammulina*, *Pleurotus*, *Tremella*, *Ganoderma* and *Trametes* (Coriolus) (Wasser and Weis, 1999).

As a result of the large numbers of scientific studies that have been conducted on medicinal mushrooms, different bioactive compounds of edible mushrooms have been discovered. These compounds (both cellular components and secondary metabolites) are responsible for several activities, such as:

- antioxidant (Mau *et al.*, 2002; Lo and Cheung, 2005; Barros *et al.*, 2007; Ferreira *et al.*, 2007; Barros *et al.*, 2008b; Ferreira *et al.*, 2009; Heleno *et al.*, 2010; Heleno *et al.*, 2012b; Pereira *et al.*, 2012); this topic will be detailed in Section 4;

- antitumour (Wasser and Weis, 1999a; Wasser, 2002; Borchers *et al.*, 2004; Lindequist *et al.*, 2005; Zaidman *et al.*, 2005; Poucheret *et al.*, 2006; Doody *et al.*, 2007; Moradali *et al.*, 2007; Zhang *et al.*, 2007; Ferreira *et al.*, 2010); this topic will be detailed in Section 4;

- antimicrobial and antiparasitic (Barros *et al.*, 2007; Barros *et al.*, 2008a; Barros *et al.*, 2008b; Rao *et al.*, 2009; Alves *et al.*, 2012);

- immunomodulatory (Chiu *et al.*, 2000, Lull *et al.*, 2005; Moradali *et al.*, 2007; Israilides *et al.*, 2008);

- antiatherogenic (Kaneda and Tokuda, 1966; Bobek and Galbavý, 1999; Yamada *et al.*, 2002; Mori *et al.*, 2008);

- hypocholesterolemic (Kaneda and Tokuda, 1966; Bobek *et al.*, 1991; Bobek *et al.*, 1994; Bobek *et al.*, 1995; Bobek and Ozdín, 1996; Bobek *et al.*, 1998; Bobek and Galbavý, 1999) and

- hypoglycemic (Wasser and Weis, 1999).

The bioactivity of Basidiomycetes (Agaricomycetes) mushrooms was confirmed by Lucas for the first time back in 1957 (Lucas, 1957). In this study, a substance from *Boletus edulis* that had a significant growth inhibitory effect against Sarcoma S-180 tumour cells was isolated. Carrying out an extensive study in 1966, Gregory and their collaborators isolated the active substances from the fruiting bodies of more than 200 Basidiomycetes (Agaricomycetes) mushroom species, and from 7000 culture media produced by applying submerge fermentation to the correspondent mushroom types with antitumour activity (Gregory, 1966).

A wide variety of pathological damage related to the ageing process (such as DNA damage, carcinogenesis and cellular degeneration) can be caused by reactive oxygen species (ROS) produced by sunlight, ultraviolet or ionizing radiation, chemical reactions and metabolic processes (Ferreira *et al.*, 2009). Furthermore, there is a vast accumulation of studies that implicate oxygen derived free radicals such as superoxide, hydroxyl radicals and high energy oxidants such as peroxynitrite as mediators of inflammation, shock and ischemia/reperfusion injury. There is also growing evidence to show that production of ROS at the site of inflammation can contribute to tissue damage. Therefore, interventions against ROS could exert beneficial effects on inflammation and shock (Halliwell and Gutteridge, 1984). In this context, several mushroom species have been studied for their antioxidant activities (Ferreira *et al.*, 2009). This will be further detailed in section 4.

Many species of mushrooms have been found to be highly potent immune system enhancers, potentiating human and other animals immunity against cancer (Borchers *et al.*, 2004; Brzin *et al.*, 2000; Wasser and Weis, 1999; Ikekawa, 2001; Poucheret *et al.*, 2006; Moradali *et al.*, 2007). At least 30 mushroom species have yielded compounds with pronounced anticancer potential in xenographs of human tumours in mice, but only a small number have been taken to the next step, which is the objective clinical assessment for anticancer potential in humans (Ferreira *et al.*, 2010). This will be further detailed in section 4.

1.3 FUNGI BIODIVERSITY IN NORTHEAST OF PORTUGAL

Recent estimates indicate the existence of approximately 1.5 million species of fungi worldwide, of which about 55,000 are mushrooms (macrofungi). This enormous diversity of the Fungi makes this kingdom the major group of organisms known. Mushrooms can be found in virtually all natural habitats and semi-natural habitats,

from rainforests to the frozen wastes of Antarctica. Yet it is in forest ecosystems that they find their ideal ecological conditions (Figure 2).

Figure 2. Northeast of Portugal [adapted from (Gaspar, 2003)].

The northeast of Portugal, due to its climatic conditions and flora diversity, is one of the European regions with a high diversity of wild edible mushrooms, some of them with great gastronomic relevance. Within the local edible species, *Lactarius deliciosus*, *Boletus edulis*, *Hydnum rufescens* and *Cantharellus cibarius* are the most important because of their high consumption by the rural population and their economic value in the markets of France and Spain (Martins *et al.*, 2002; Baptista *et al.*, 2003).

Several wild mushrooms species collected in this region have already been identified and further characterized (Table 1).

Table 1. List of the wild mushroom species from Northeast of Portugal already studied.

Mushroom species	Ecology/ Edibility	Aspects studied				Ref.
		Nutritional value	Chemical characterization[a]	Antioxidant activity	Antimicrobial activity	
Agaricus arvensis	S/E	x	x	x	X	Barros *et al.*, 2007; Alves *et al.*, 2012
Agaricus bisporus	S/E	x	x	x	x	Barros *et al.*, 2008c; Alves *et al.*, 2012
Agaricus campestris	S/E	x	x	x		Pereira *et al.*, 2012

Mushroom species	Ecology/ Edibility	Aspects studied				Ref.
		Nutritional value	Chemical characterization[a]	Antioxidant activity	Antimicrobial activity	
Agaricus comtulus	S/E	x	x	x		Pereira *et al.*, 2012
Agaricus lutosus	S/E	x	x	x		Pereira *et al.*, 2012
Agaricus silvaticus	S/E	x	x	x	x	Barros *et al.*, 2008c
Agaricus silvicola	S/E	x	x	x	x	Barros *et al.*, 2008c
Amanita caesarea	M/C		x	x		Reis *et al.*, 2011a
Amanita muscaria	M/NE		x	x		Reis *et al.*, 2011a
Amanita pantherina	M/NE		x	x		Reis *et al.*, 2011a
Amanita porphyria	M/NE		x	x		Reis *et al.* 2011b
Amanita umbrinolutea	M/E	x	x	x		Pereira *et al.*, 2012
Boletus aereus	M/C	x	x	x		Heleno *et al.*, 2011
Boletus armeniacus	M/E	x	x	x		Pereira *et al.*, 2012
Boletus citrinoporus	M/NE		x	x		Reis *et al.* 2011b
Boletus edulis	M/E	x	x	x		Heleno *et al.*, 2011
Boletus erythropus	M/E	x	x	x		Grangeia *et al.*, 2011
Boletus fragrans	M/E	x	x	x		Grangeia *et al.*, 2011
Boletus impolitus	M/E	x	x	x		Pereira *et al.*, 2012
Boletus purpureus	M/NE	x	x	x		Heleno *et al.*, 2011
Boletus reticulates	M/E	x	x	x		Heleno *et al.*, 2011
Boletus rhodoxanthus	M/NE	x	x	x		Heleno *et al.*, 2011
Boletus satanas	M/NE		x	x		Heleno *et al.*, 2011
Bovista aestivalis	S/E	x	x	x		Pereira *et al.*, 2012
Bovista nigrescens	S/E	x	x	x		Pereira *et al.*, 2012
Calvatia utriformis	S/E	x	x	x		Grangeia *et al.*, 2011
Cantharellus cibarius	M/E	x	x	x	x	Barros *et al.*, 2008a; Alves *et al.*, 2012
Chlorophyllum rhacodes	S/C	x	x	x		Pereira *et al.*, 2012
Chroogomphus fulmineus			x	x		Reis *et al.*, 2011a
Clavariadelphus		x	x	x		Pereira *et*

Mushroom species	Ecology/ Edibility	Aspects studied				Ref.
		Nutritional value	Chemical characterization[a]	Antioxidant activity	Antimicrobial activity	
pistillaris						*al.*, 2012
Clavariadelphus truncates		x	x	x		Pereira *et al.*, 2012
Clitocybe costata		x	x	x		Pereira *et al.*, 2012
Clitocybe gibba		x	x	x		Pereira *et al.*, 2012
Clitopilus prunulus	S/E	x	x	x		Grangeia *et al.*, 2011
Collybia fusipes	S/NE		x	x		Reis *et al.* 2011b
Coprinopsis atramentaria	S/E		x	x		Heleno *et al.*, 2012b
Cortinarius anomalus			x	x		Reis *et al.*, 2011a
Cortinarius collinitus			x	x		Reis *et al.*, 2011a
Cortinarius glaucopus		x	x	x		Heleno *et al.*, 2009
Cortinarius violaceus			x	x		Reis *et al.*, 2011a
Fistulina hepatica	P/E		x	x	x	Heleno *et al.*, 2009; Alves *et al.*, 2012
Flammulina velutipes		x	x	x		Pereira *et al.*, 2012
Fomitopsis pinicola	S/NE		x	x		Reis *et al.*, 2011b
Ganoderma lucidum	P or S/E		x	x		Heleno *et al.*, 2012a
Hebeloma sinapizans	M/NE		x	x		Reis *et al.*, 2011b
Hygrophoropsis aurantiaca		x	x			Heleno *et al.*, 2009
Hygrophorus chrysodon		x	x	x		Pereira *et al.*, 2012
Hygrophorus pustulatus	M/E	x	x	x		Grangeia *et al.*, 2011
Hypholoma capnoides		x	x			Heleno *et al.*, 2009
Inocybe splendens	S/NE		x	x		Reis *et al.*, 2011b
Laccaria laccata		x	x			Heleno *et al.*, 2009
Lactarius bertillonii	M/NE		x	x		Heleno *et al.*, 2012b
Lactarius deliciosus	M/E	x	x	x	x	Barros *et al.*, 2007; Alves *et al.*, 2012
Lactarius hepaticus	M/NE		x	x		Reis *et al.*, 2011b
Lactarius quietus		x	x			Reis *et al.*, 2011a
Lactarius	M/E	x	x		x	Heleno *et*

Mushroom species	Ecology/ Edibility	Aspects studied				Ref.
		Nutritional value	Chemical characterization[a]	Antioxidant activity	Antimicrobial activity	
salmonicolor						*al.*, 2009; Alves *et al.*, 2012
Lactarius vellereus	M/NE		x	x		Heleno *et al.*, 2012b
Lactarius volemus		x	x			Reis *et al.*, 2011a
Lentinus tigrinus	S/NE		x	x		Reis *et al.*, 2011b
Lepista inversa		x	x			Heleno *et al.*, 2009
Lepista nuda	S/E	x	x	x	x	Barros *et al.*, 2008a; Alves *et al.*, 2012
Leucoagaricus leucothites		x	x	x		Pereira *et al.*, 2012
Leucopaxillus giganteus	S/E	x	x	x	x	Barros *et al.*, 2007; Alves *et al.*, 2012
Lycoperdon echinatum	S/E	x	x	x		Grangeia *et al.*, 2011
Lycoperdon molle		x	x	x	x	Barros *et al.*, 2008a
Lycoperdon perlatum		x	x	x	x	Barros *et al.*, 2008a
Lycoperdon umbrinum		x	x	x		Pereira *et al.*, 2012
Lyophyllum decastes	S/E	x	x	x		Grangeia *et al.*, 2011
Macrolepiota excoriata	S/E	x	x	x		Grangeia *et al.*, 2011
Mycena rosea	S/E				x	Alves *et al.*, 2012
Paxillus involutus	M/NE		x	x		Reis *et al.*, 2011c
Piptoporus betulinus	P/NE		x	x		Reis *et al.*, 2011b
Pisolithus arhizus	M/E		x	x		Reis *et al.*, 2011c
Pluteus murinus	S/NE		x	x		Reis *et al.*, 2011b
Ramaria aurea	M/E	x	x	x		Pereira *et al.*, 2012
Ramaria botrytis	M/E	x	x	x	x	Barros *et al.*, 2008a; Alves *et al.*, 2012
Rhodotus palmatus	S/UK		x	x		Heleno *et al.*, 2012b
Russula cyanoxantha	M/E	x	x	x		Grangeia *et al.*,

Mushroom species	Ecology/ Edibility	Aspects studied				Ref.
		Nutritional value	Chemical characterization[a]	Antioxidant activity	Antimicrobial activity	
						2011
Russula delica	M/E	x	x		x	Heleno *et al.*, 2009; Alves *et al.*, 2012
Russula emetica	M/NE		x	x		Reis *et al.*, 2011b
Russula olivacea	M/E	x	x	x		Grangeia *et al.*, 2011
Russula sardonia	M/NE		x	x		Reis *et al.*, 2011a
Sarcodon imbricatus	M/E	x	x	x	x	Barros *et al.*, 2007; Alves *et al.*, 2012
Suillus luteus	M/E		x	x		Reis *et al.*, 2011a
Suillus mediterraneensis	M/E		x	x		Heleno *et al.*, 2009
Suillus variegatus	M/E	x	x	x		Pereira *et al.*, 2012
Tricholoma imbricatum	S/E	x	x			Heleno *et al.*, 2009
Tricholoma portentosum	M/E	x	x	x	x	Barros *et al.*, 2007; Alves *et al.*, 2012
Tricholoma ustale	M/NE		x	x		Reis *et al.*, 2011a
Xerocomus chrysenteron	M/E		x	x		Heleno *et al.*, 2012b

[a]*Compounds identified: phenolic compounds, tocopherols, ascorbic acid, β-carotene.*
M - Mycorrhizal; S – Saprotrophic; P – Parasitic; E – Edible; NE – Not edible; C – Choice; UK – Unknown.

The popularity of mushrooms in the northeast of Portugal and their increasing exportation to countries like Spain, France and Italy, justify the further study of these wild species. Thus, knowledge of the properties of these species of wild mushrooms should be well documented and disseminated, in order to allow better management of their potential use and the conservation of their *habitats* (Ferreira, 2011).

2. ANTIOXIDANT ACTIVITY

All aerobic organisms require molecular oxygen as electron acceptor for the production of energy.

In the metabolism of aerobic biological systems a continuous production of (ROS) and reactive nitrogen species (RNS) occurs. Other possible sources of ROS are air pollutants, tobacco or smoke, UV radiation and a diet high in polyunsaturated fatty acids (Goetz and Luch, 2008). When this balance tends to produce free radicals, the body is under oxidative stress (Figure 3). Free radicals in excess can oxidize and damage cellular lipids, proteins and DNA, inhibiting their normal function and leading to various diseases such as cancer (Valko *et al.*, 2007; Ferreira *et al.*, 2009). The majority of the targets of oxidative stress at the cellular level are the cell membrane (causing inactivation of enzymes and changes in transmembrane transport), the cytoplasm and its components (proteins and lipids) and, mostly, the genetic material (causing mutagenesis, carcinogenesis and aging).

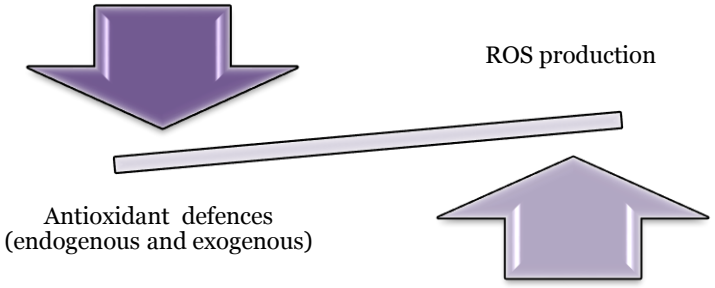


Figure 3. Schematic representation of the balance between production of free radicals and antioxidant defences.

2.1 OXIDATIVE STRESS

The expression of free radicals produced from natural metabolism of aerobic cells is mostly ROS. Once produced, most of the free radicals are neutralized by cellular antioxidant defences (enzymes and non-enzymatic molecules) (Figure 4). Beneficial effects of ROS occur at low or moderate concentrations and involve cellular physiological roles of signalization and regulation (Freidovich, 1999; Fang *et al.*, 2002).

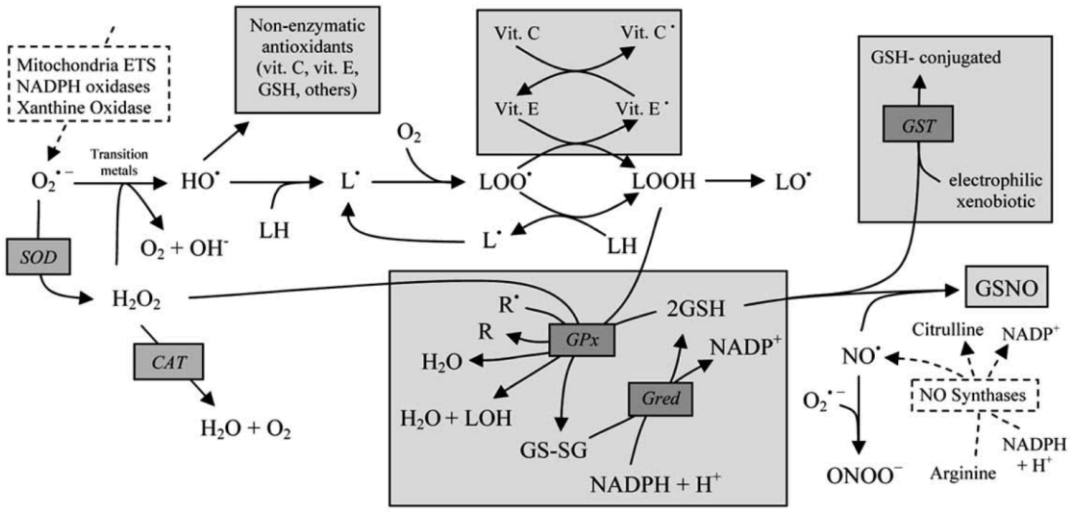


Figure 4. Overview of the main reactions involving reactive Oxygen species (ROS) / reactive Nitrogen species (RNS), and major endogenous enzymatic and non-enzymatic antioxidant defences in the cell. Representative endogenous enzymatic and non-enzymatic antioxidant defences in the cell [adapted from Ferreira *et al*. 2009)]. Representative endogenous sources (traced rectangles). Main antioxidant defences (shaded rectangles) and the enzymes involved (in italic).

As can be seen in Figure 4, the most representative endogenous sources of ROS/RNS include: Mitochondrial ETS (Electron transport system), NADPH oxidases, Xanthine oxidase for ROS and NO synthases for RNS. The addition of one electron to molecular Oxygen forms the superoxide anion (O2 ·-), which is considered the "primary" ROS. Superoxide anion is mostly produced in mitochondria, due to a small but continuous "leak" of the electrons in the mitochondrial electron transport system (ETS). These electrons generate superoxide anion instead of reducing oxygen to water. Superoxide anion can also be produced by different endogenous enzymatic systems present in the cell like NADPH oxidases and xanthine oxidase. O2·- is not a very active radical, it can interact with other molecules generating what are considered as "secondary" ROS, such as hydrogen peroxide (H2O2) and hydroxyl radical (OH·). Hydroxyl radical has a very short life time but is considered to be the most toxic among all ROS. Hydroxyl radical is the neutral form of the hydroxide ion and it is formed by an electron transfer from transition metals to H2O2, and interacts with biomolecules immediately after generation (Ferreira *et al.*, 2009).

Maintenance of the equilibrium between free radicals production and antioxidant defences is an essential condition for the normal function of organisms (Valko *et al.* 2007).

Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage (Machlin and Bendich, 1987).

Disturbances in this normal redox state can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids and DNA, leading to their modification and inhibiting their normal function (Fu *et al.*, 1998; Ridnour *et al.*, 2005; Valko *et al.* 2007).

In humans, oxidative stress might have natural causes such as extreme exercise or inflammation processes, or non-natural causes such as the presence of xenobiotics in the organism or situations related to several diseases, such as several kinds of cancer (Valko *et al.* 2006), diabetes (Valko *et al.* 2007), cirrhoses (Wei *et al.*, 2004), cardiovascular diseases (atherosclerosis, heart failure, myocardial infarction) (Shah and Channon, 2004), neurological disorders (Parkinson's and Alzheimer's diseases) (Moreira, *et al.*, 2008), fragile X syndrome and chronic fatigue syndrome. Nevertheless, short-term oxidative stress may also be important in the prevention of aging by induction of a process named mitohormesis (Barja, 2004).

Considering that 70% of the chronical diseases and related costs can be prevented, the knowledge about ROS and about the control of their overproduction is crucial (Ferreira *et al.*, 2009). This control can be achieved by the maintenance of good levels of antioxidants and free radicals scavengers, increasing the quality of diet (higher consumption of vegetables, leguminous and fruits) or avoiding behaviours that lead to a higher production of ROS, such as tobacco, excessive exposure to environmental polluents and xenobiotics (Lachance *et al.*, 2001).

Nevertheless, ROS can be beneficial, as they are used by the immune system as a way to attack and kill pathogens. These species are also used in cell signaling (Barja, 2004).

2.2 ENDOGENOUS ANTIOXIDANT DEFENCES

As mentioned previously, antioxidant defence systems protect cellular homeostasis from oxidative disruption by reactive molecules generated through the reduction of molecular oxygen. The efficient functionality of these mechanisms requires the concerted action of the individual systems. These defence systems also have to be in concert with the

components responsible for the repair processes of oxidatively damaged molecules in order to maintain the cell integrity.

The endogenous antioxidant defences may be enzymatic or nonenzymatic. The enzymatic antioxidant defences are numerous and are spread throughout the body. Examples of these defences are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (Gred). Among the non-enzymatic antioxidant defences are compounds such as glutathione (GSH), α-tocopherol (Vit. E) and ascorbic acid (Vit. C) (Figure 3; Ferreira *et al.*, 2009).

SOD converts $O_2^{\cdot-}$ into H_2O_2, which is then detoxified to water either by CAT in the peroxysomes or by GPx in the mitochondria, cytosol or nucleus. GPx are a group of selenoenzymes that require Selenium on their biosynthesis. Another important enzyme is Gred, which regenerates GSH that is used as a hydrogen donor by GPx. GPx can also transform hydroperoxide lipids into alcohols (LOH). Glutathione (GSH) is a low molecular weight tripeptide composed of glutamate, cysteine, and glycine being the main intracellular redox buffer. The capacity of GSH to regenerate the most important antioxidant molecules is linked with the redox state of the glutathione disulphide/glutathione (GSSG/GSH) couple. GSH effectively scavenges ROS ($HO^{\cdot}$, H_2O_2, $LOO^{\cdot}$ and $ONOO^{-}$) either directly or indirectly as a cofactor of several detoxifying enzymes, e.g. GPx, GST, among others. In the neutralization process of ROS, GSH is oxidized to glutathione disulphide (GS-SG), which can be further reduced to two GSH by the enzyme Gred. GSH is also able to regenerate other antioxidant molecules such as vitamins C and E. GSH can also react with a variety of electrophilic xenobiotics in reactions catalysed by glutathione-Stransferases (GST) generating products with higher solubility and thus easier to eliminate. GSH can also neutralize $NO^{\cdot}$, resulting in the formation of S-nitrosoglutathione (GSNO). Vitamin E is a liposoluble vitamin present in the membranes thus playing an important role in the prevention of lipid peroxidation. Among the eight forms of vitamin E, β-tocopherol is the most active form in humans. ROS (hydroxyl and peroxyl radicals, etc.) react with vitamin E, generating a poorly reactive phenolic radical (vit. $E^{\cdot}$). Vitamin C then reacts with vit. $E^{\cdot}$ producing vitamin C radical (vit. $C^{\cdot}$) and regenerating vitamin E. Both radicals (vit. $E^{\cdot}$ and vit. $C^{\cdot}$) are poorly reactive species because of its unpaired electron.

In addition to the endogenous defences, there is a range of natural or synthetic molecules with antioxidant properties that may be an exogenous system of defence. A multitude of natural antioxidants have already been isolated from different kinds of plant

materials such as oilseeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs (Ferreira *et al.*, 2009).

Some natural products with antioxidant activity may be useful to aid the system endogenous defences, being used as nutraceuticals.

2.3 EXOGENOUS ANTIOXIDANT DEFENCES: THE CASE STUDY OF PHENOLIC ACIDS

Among the biologically active substances present in mushrooms, phenolic compounds have attracted much attention due to their very good properties as antioxidant, anti-inflammatory or antitumour agents, among others (Puttaraju *et al.*, 2006).

Phenolic compounds are aromatic hydroxylated compounds, possessing one or more aromatic rings with one or more hydroxyl groups. They include a large number of subclasses, such as flavonoids, phenolic acids, including hydroxybenzoic acids and hydroxycinnamic acids, stilbenes, lignans, tannins, and oxidised polyphenols, displaying an enormous diversity of structures (D'Archivio *et al.*, 2010; Cote *et al.*, 2010).

Phenolic acids within mushrooms (Figure 5 and 6) are strong antioxidants and may alleviate oxidative stress by quenching or neutralising reactive species, thereby reducing cellular damage or death (Andreasen *et al.*, 2001; Zhou and Yu, 2004). Besides acting as antioxidants, these compounds can be beneficial to health by chelating metal ions (Liyana-Pathirana and Shahidi, 2006), stimulating antioxidative (Moore *et al.*, 2006) and detoxifying (Yoshioka *et al.*, 1995) enzymes, and inhibiting transcription factors that initiate and promote tumour cell growth (Yoshioka *et al.*, 1995; Natarajan *et al.*, 1996).

Benzoic acid derivatives	Substitution				
	X	R^1	R^2	R^3	R^4
p-Hydroxybenzoic	COOH	H	H	OH	H
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

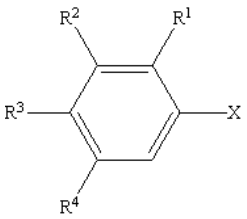


Figure 5. Chemical structure of the benzoic acid derivatives found in mushrooms [adapted from (Ferreira et al., 2009.)].

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	CH_3 O	OH	H
Sinapic	CH_3O	H	CH_3O	OH	CH_3O
3-*O*-caffeoylquinic	*	H	OH	OH	H
4-*O*-caffeoylquinic	*	H	OH	OH	H
5-*O*-caffeoylquinic	*	H	OH	OH	H

* The carboxylic group is esterified with quinic acid

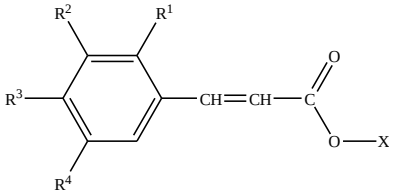


Figure 6. Chemical structure of the cinnamic acid derivatives found in mushrooms [adapted from (Ferreira et al., 2009)].

Phenolic compounds can be classified as free radical inhibitors (chain breakers), peroxide decomposers, metal inactivators or oxygen scavengers. Biological antioxidant capacity can be measured by controlling the inhibition of induced lipid oxidation (Tsukihara *et al.*, 2008). These methods induce the auto-oxidation of linoleic acid or low-density lipoproteins (LDL) by Cu(II) or an azo initiator and control the formation of conjugated diene peroxides.

Free radicals, generated by the initiator, react with oxygen species yielding peroxide radicals which attack the lipids to form the conjugated diene peroxides. Therefore, the scavenging of free radicals or peroxide radicals by an antioxidant agent may avoid the lipid oxidation in biological systems (Palacios *et al.*, 2011). Gallic acid (3,4,5-tri-hydroxybenzoic acid) has a greater antioxidant activity than protocatechuich acid (3,4-dihydroxybenzoic acid), apparently due to the presence of more hydroxyl groups (Cuvelier *et al.*, 1992; Palacios *et al.*, 2011).

Substitutions with methoxyl groups (electron donors) in a position *ortho* to the hydroxyl group increases the antioxidant activity by stabilizing the phenoxyl radical, sinapic acid> ferulic acid> *p*-coumaric acid and syringic acid> vanillic acid> *p*-hydroxybenzoic acid. However, the antioxidant activity of methoxyl groups is always inferior to the presence of hydroxyl groups: e.g. ferulic acid <caffeic acid and vanillic acid <protocatechuich acid (Cuvelier *et al.*, 1992).

The esterification with quinic acid decreases the antioxidant activity, chlorogenic acid <caffeic acid. The allyl group present in cinnamic acid (CH = CHCOOH) derivatives appears to increase the antioxidant activity compared to benzoic acid (COOH) derivatives: caffeic acid> protocatechuich acid, sinapic acid> syringic acid, ferulic acid> vanillic acid

and *p*-coumaric acid> *p*-hydroxybenzoic acid. The double bond C = C appears to participate in the stabilization of the phenoxyl radical (Cuvelier *et al.*, 1992).

The individual profile of phenolic compounds in wild mushrooms has been obtained by high-performance liquid chromatography coupled to photodiode array detector/electrospray ionization mass spectrometry (HPLC-DAD/ESI-MS) (Barros *et al.*, 2009), with UV detector (HPLC-UV) or Gas chromatography coupled to mass spectrometry (GC-MS) (Ferreira *et al.*, 2009).

3. CANCER CELLS GROWTH

Cancer has become a major public health problem worldwide with an estimated prevalence of about 3%, increasing to 15% in individuals aged over 65 years. Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. The burden of cancer is increasing in economically developing countries as a result of population aging and growth as well as, increasingly, an adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and "westernized" diets (Jemal *et al.*, 2011). Moreover, cancer related deaths are projected to increase to over 11 million in 2030 (World Health Organization, 2010).

About 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008 worldwide, with 56% of the cases and 64% of the deaths in the economically developing world. Breast cancer in females and lung cancer in males are the most frequently diagnosed cancers and the leading cause of cancer death for each sex in both economically developed and developing countries, except that lung cancer is preceded by prostate cancer as the most frequent cancer among males in economically developed countries (Jemal *et al.*, 2011).

Many years of research have revealed that cancer results from genetic alterations in cancer cells which confer advantages to those cells, such as capacity of unlimited division and evasion of cell death. Hanahan and Weinberg (2012), claim that the most fundamental trait of cancer cells involves their ability to sustain chronic proliferation.

The main approaches to cancer treatment are surgical excision, radiotherapy and chemotherapy. The use of anticancer drugs, as part of a treatment strategy to cancer, has greatly improved the overall prognosis of cancer patients (Maser and DePinho, 2002; Finkel *et al.*, 2007). Therefore, efforts to discover new medicines for treating cancer and to predict their clinical activity are essential (Sharma *et al.*, 2010).

In normal cells, growth, division and differentiation are highly regulated processes. Some signaling molecules—called growth factors—promote cell division. Other signaling molecules cause cells to stop growing. Many signaling molecules, including growth factors and growth inhibitors, bind to receptors on the surface of the cell. In many cases, these receptors must interact with one another, or dimerize, before they can become fully activated (Walensky, 2006).

Unlike normal cells, cancer cells display uncontrolled growth control, so they can ignore signals to stop growing. Some cancer cells can make their own growth factors. These growth factors travel to the outside of the cell, where they interact with and activate the cancer cell's growth factor receptors. Some cancer cells make more growth factor receptors than normal cells—this is called overexpression. Cancer cells with overexpressed growth factor receptors may be stimulated to grow when growth factors are present at levels that would be too low to stimulate growth of normal cells. This is because by having more receptors available increases the chances that a growth factor will find its receptor. Other cancer cells may have mutations in the genes that code for growth factor receptors. Some of these mutations result in the formation of dysfunctional receptors, which remain in the "ON" position for growth even when no growth factor is present (Barinaga, 1996; Walensky, 2006).

Because cancer cells display uncontrolled cell growth, most anticancer therapy is aimed at increasing levels of cell death and reducing cellular proliferation (Schreader and Nambu, 2004; Hague and Verkhratsky, 2009).

3.1 SCREENING ASSAYS OF INHIBITION OF CELL GROWTH

Practical endpoints for microplate cytotoxicity assays did not exist until the time when the US National Cancer Institute (NCI) model was conceived, in the late 1980s (Shoemaker, 2006). Indeed, the tritiated thymidine incorporation assay that was widely used until then for growth-inhibition studies was prohibitively expensive for large-scale screening.

One of the presently existing assays for evaluation of cell growth inhibitory activity is the Alamar blue assay. Alamar blue is a sensitive oxidation–reduction indicator that fluoresces and changes color upon reduction by living cells. The reduction of Alamar blue is believed to be mediated by mitochondrial enzymes (O'Brien *et al.*, 2000). However, Gonzalez and Tarloff (2001) suggest that cytosolic and microsomal enzymes contribute to the reduction of Alamar blue.

The MTT assay is another presently well-documented cell metabolic assay and has been modified by several investigators since it was first developed by Mosmann in 1983 (Hamid *et al.*, 2004). This assay is based on the cleavage of the yellow tetrazolium salt, MTT, by mitochondrial enzymes, to form a soluble blue formazan product. The amount of

formazan produced is directly proportional to the number of living cells present during MTT exposure (Sylvester, 2011).

Since the MTT assay is rapid, convenient, and economical, it has become a very popular technique for quantification of metabolically active cells in culture (Vistica, 1991; Sylvester, 2011). However, various parameters have been identified that may affect cellular metabolism, which significantly modify MTT-specific activity and can result in calculated false high or low cell growth results (Sylvester, 2011).

Therefore, it is essential to establish the assay parameters with the proper controls for each cell line and/or drug treatment, in order to optimize the assay conditions and minimize confounding effects. These parameters should include determining appropriate cell densities, culture medium, optimal concentrations and exposure times for MTT, fresh culture medium at the time of assay initiation to avoid nutrient depletion, and controlling the drug treatment effects that may influence cellular metabolism. A limitation of the MTT assay is that the formazan product is insoluble in aqueous medium, so a step is required to solubilize the formazan before reading the optical density. To circumvent this, a new tetrazolium assay was developed based on a reagent designated XTT (Vistica, 1991).

The XTT cell growth assay was first described by Scudiero (1988), as an effective method to measure cell growth and drug sensitivity in tumour cell lines. XTT is a colourless or slightly yellow compound that when reduced becomes brightly orange. This color change is accomplished by breaking apart the positively-charged quaternary tetrazole ring (Berridge *et al.*, 2005). The formazan product of XTT reduction is soluble and can be used in real-time assays. XTT is thought to be excluded from entering cells by its net negative charge (Berridge *et al.*, 2005). Considerable evidence suggests that XTT dye reduction occurs at the cell surface facilitated by trans-plasma membrane electron transport. Mitochondrial oxidoreductases are thought to contribute substantially to the XTT response, with their reductants being transferred to the plasma membrane. It has been proposed that XTT assays actually measure the pyridine nucleotide redox status of cells (Marshall *et al.*, 1999; Berridge *et al.*, 2005). The XTT assay served as an efficient bioassay tool for the isolation of natural products with anticancer potential and provided the basis for the NCI anti-HIV drug screen (Sausville and Shoemaker, 2001).

Alternative approaches were investigated, including a methylene blue assay, and a method that used sulphorhodamine B (SRB) was finally selected by NCI for use in the screening of tumour cell growth.

The SRB assay, based on the measurement of cellular protein content, is used for cell density determination (Keepers *et al.*, 1991). This method has been optimized for the toxicity screening of compounds in cell lines, in a 96-well plate. The results are linear over a 20-fold range of cell numbers and the sensitivity is comparable to those of fluorometric methods. This method not only allows a large number of samples to be tested within a few days, but also only requires simple equipment and inexpensive reagents. The assay also has the advantage of allowing some degree of differentiation between cell kill from cell growth inhibition (Skehan *et al.*, 1990).

The SRB assay is therefore an efficient and highly cost-effective method for screening compounds or natural products (Skehan *et al.*, 1990; Vichai and Kirtikara; 2006). This assay proved to be robust and feasible for medium-scale screening (Monks *et al.*, 1997).

The application of the SRB assay to the pilot-scale screening of test sets of compounds, including approved anticancer drugs, led to the recognition of the profiles of cell line sensitivity and resistance, reflecting mechanisms of growth inhibition and cell killing (Shoemaker, 2006).

3.2 CELL PROLIFERATION AND CELL DEATH

Cell proliferation

The core of cellular proliferation is the cell cycle division. This process includes a series of events occurring in an ordered sequence, which result in genome duplication and segregation to daughter cells following division (Figure 7). Cell cycle is comprised of two active phases: the S phase, in which DNA synthesis/chromosome replication occurs (Alberts *et al.*, 2002), and the M phase (mitosis), in which separation of the chromosomes followed by cell division (cytokinesis) occurs. These processes are separated by two gaps (G1 and G2).

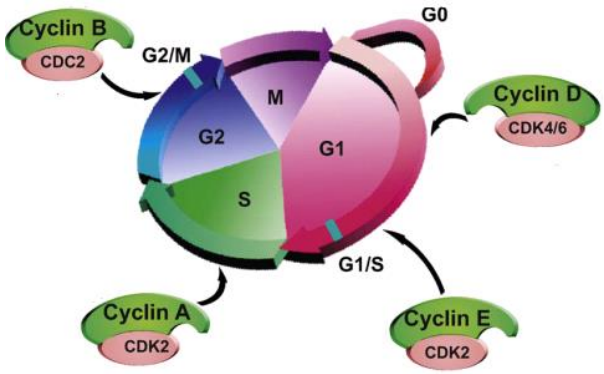


Figure 7. Simple representations of cell cycle and some of its regulators [adapted from (Wang *et al.*, 2009)]. The purple circle represents phases G1, S, G2, and M of the cell cycle. The cyclin/cyclin-dependent kinases (CDK) complexes present on each phase are represented surrunded by a circle.

During the G2 phase biosynthesis occurs, mainly the production of microtubules, which are required for the process of mitosis. The relatively brief M phase consists of nuclear division (karyokinesis), followed immediately by cytokinesis. Finally, there is a fifth state, G0 (also known as quiescence) into which the cell may reversibly exit from G1, if it is deprived of the appropriate growth-promoting signals (Morgan, 2007).

Progression through the cell-division cycle is driven by activation and inactivation of cyclin-dependent kinases (CDKs), which trigger the transition to the subsequent phases of the cycle. CDKs are small serine/threonine protein kinases that require association with a cyclin subunit for their activation (Besson *et al.*, 2008).

Many levels of regulation exist to impose tight control over cell-cycle progression (Schwartz and Shah, 2005). Such regulation involves controlled expression and destruction of cyclins, activating and inhibitory phosphorylation and dephosphorylation of the CDKs, and expression or destruction of inhibitory proteins that associate with CDKs, or CDK/cyclin complexes (Figure 7). Two families of genes, the cip/kip family (such as p21 or p27) and the INK4a/ARF (inhibitor of kinase 4/alternative reading frame; such as p16) prevent the progression of the cell cycle, and are therefore accepted as being tumour suppressors (Dynlacht, 1997; Fung and Poon, 2005; Morgan, 2007).

The regulation of cell cycle ensures quality in cellular DNA replication and division. Any deregulation in this mechanism will lead to alterations in cell cycle progression or in the replication of DNA that may be mutated or damaged, or even in uncontrolled proliferation, thus contributing to tumour development (Paulovich *et al.*, 1997).

Several anticancer drugs bind directly to DNA (for example, intercalating agents), interfere with DNA synthesis (for example, antimetabolites) or with microtubule polymerization/depolymerisation (vinka alkaloids and taxol, respectively), thus interfering with the cell division and blocking cell growth (De Falco and De Luca, 2010).

Cell death

Programmed cell death is a central mechanism controlling multicellular development, and is involved in a variety of biological events. These include morphogenesis, maintenance of tissue homeostasis or elimination of harmful cells. Programmed cell death leads to deletion of entire structures, sculpts specific tissues (by ablating fields of cells) and regulates the number of neurons in the nervous system (Lodish *et al.*, 2000; Sun and Peng, 2009).

Cellular interactions regulate cell death in two fundamentally different ways. Most cells in multicellular organisms, if not all, require signals to stay alive. In the absence of such survival signals, frequently referred to as trophic factors, cells activate a "suicide" program. On the other hand, in some developmental contexts, including the immune system, specific signals induce a "suicide" program that kills cells. Independently of the reason for the cells to commit suicide (lack of survival signals or killing signals from other cells), recent studies suggest that the death may be mediated by a common molecular pathway.

As mentioned earlier, cell death is a critical process in development and homeostasis of normal organisms, but is also important in certain pathological conditions, including cancer. Indeed, alterations in the apoptotic machinery, including alterations in its regulators, have been described in several human cancers. Moreover, transformed cells usually acquire defects in the apoptotic pathways therefore having a survival advantage (Mashima and Tsuruo, 2005).

For several years it had been believed that the mechanism of cell death induced by chemotherapy was confined solely to apoptosis (Meier *et al.*, 2000; Sun and Peng, 2009). However, accumulating evidence suggested that tumour cellular response to chemotherapy includes other modes of cell death (Okada and Mak, 2004). Indeed, there are four described modes of cell death that can be induced by chemotherapy: apoptosis, necrosis, mitotic catastrophe and autophagy (Ashkenazi, 2002; Galluzzi *et al.*, 2007). Senescence, a form of permanent growth arrest, is not a cell death mechanism but is

considered of significant importance as a response of cancer cells to chemotherapy (Ricci and Zong, 2006). These five kinds of cellular responses are classified based on biochemical and morphological features that are present in the dying cells (Galluzzi *et al.*, 2007).

One of the most studied forms of programmed cell death is marked by a well-defined sequence of morphological changes, collectively referred to as apoptosis, a Greek word that means "dropping off" or "falling off" as in leaves from a tree. Dying cells shrink and condense and then fragment, releasing small membrane-bound apoptotic bodies, which generally are phagocytosed by other cells (Figure 8). This type of cell death is under the control of a strict cellular program. Thus, apoptotic cell death acts as part of a quality control and repair mechanism by elimination of unwanted, genetically damaged, or senescent cells, and as such is critically important for the development of organisms. Highly conserved in both plants and animals, it is also the cell death mechanism best characterised at both genetic and biochemical levels (Sun and Peng, 2009).

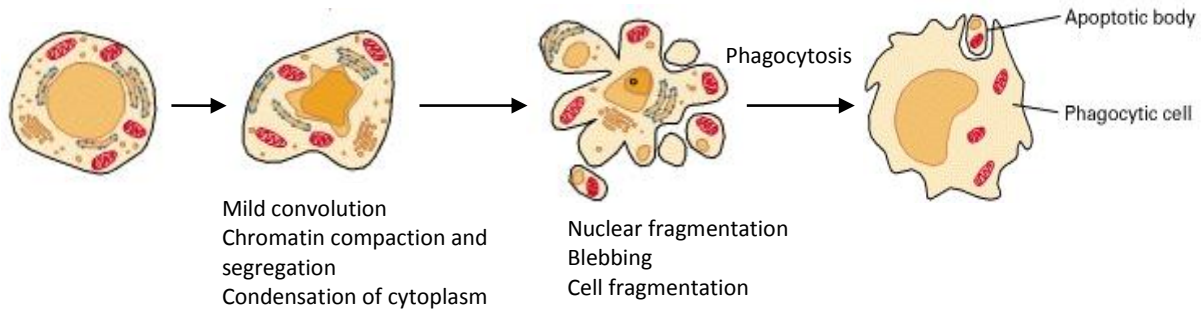


Figure 8. Schematic drawings illustrating the progression of morphologic changes observed in apoptotic cells [adapted from (Kuby, 1997)]. Early in apoptosis, dense chromosome condensation occurs along the nuclear periphery. The cell body also shrinks, although most organelles remain intact. Later, both the nucleus and cytoplasm fragment, forming apoptotic bodies. These are phagocytosed by surrounding cells.

Activation of apoptosis signaling following treatment with cytotoxic drugs may happen through the activation of the mitochondrial pathway (also named as intrinsic pathway) or the death receptors pathway (also named as extrinsic pathway) (Figure 9; Debatin, 2004).

As shown in Figure 9, both pathways will converge in the activation of caspases, cysteine proteases that are synthesized as inactive zymogens and are activated by proteolytic breakdown (Fulda and Debatin, 2004).

In the extrinsic pathway, triggering of cell surface death receptors of the tumour necrosis factor (TNF) receptor superfamily, including CD95 and TNF-related apoptosis-inducing ligand (TRAIL)-R1/-R2, results in rapid activation of the initiator caspase 8 after its recruitment to a trimerized receptor-ligand complex (DISC) through the adaptor molecule Fas-associated death domain protein (FADD). In the intrinsic pathway, stress-induced apoptosis results in perturbation of mitochondria and the ensuing release of proteins, such as cytochrome c, from the inter-mitochondrial membrane space. The release of cytochrome c from mitochondria is regulated in part by Bcl2 family members (Adams and Cory, 2007), which comprises anti-apoptotic (e.g. Bcl2/ Bcl-XL/Mcl1) and pro-apoptotic (e.g. Bax, Bak and tBid) members (Marshall *et al.*, 1999). Once released, cytochrome c binds to apoptotic protease-activating factor 1 (Apaf1), which results in formation of the Apaf1–caspase 9 apoptosome complex and activation of the initiator caspase 9. The activated initiator caspases 8 and 9 then activate the effector caspases 3, 6 and 7, which are responsible for the cleavage of important cellular substrates resulting in the classical biochemical and morphological changes (Adams and Cory, 2007).

A role for p53 in apoptosis is well known and accepted. p53 is considered to be a key guardian of the genome. It senses DNA damage and in response induces a transient growth arrest, allowing DNA repair or, in the case of extensive damage, promotes irreversible growth arrest (senescence) or programmed cell death (apoptosis) (Meek, 2009; Zuckerman *et al.*, 2009).

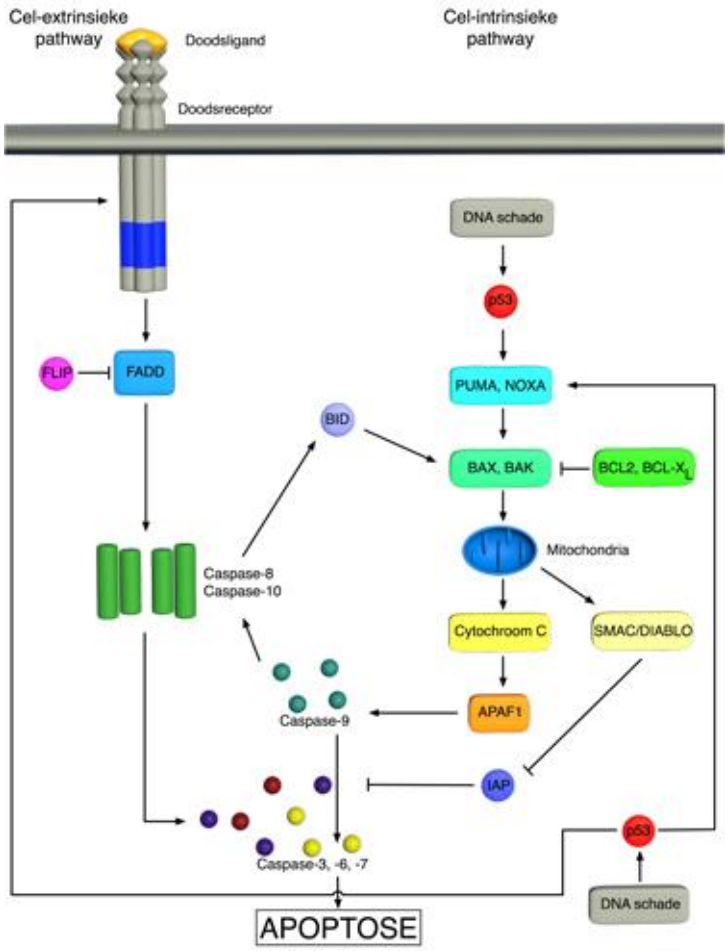


Figure 9. Apoptosis: the extrinsic and intrinsic pathways to caspase activation [adapted from (Danial and Korsmeyer, 2004)]. Two major apoptotic pathways are illustrated: one activated via death receptor activation (extrinsic) and the other by stress-inducing stimuli (intrinsic).

p53 is regarded as a central player in tumour suppression, as it controls programmed cell death (apoptosis) as well as cellular senescence. While apoptosis eliminates cells at high risk for oncogenic transformation, senescence acts as a barrier to tumourigenesis by imposing irreversible cell cycle arrest. p53 can act directly or indirectly at multiple levels of the tumour suppression network by invoking a myriad of mechanisms. p53 induces the extrinsic and intrinsic apoptotic pathways at multiple steps to ensure an efficient death response.

p53 response involves transcriptional activation or repression of target genes, as well as some recently identified microRNAs, and transcription-independent functions (Figure 10). Importantly, p53 loss of function is often found in tumours. Therefore, therapeutic strategies aimed at reactivation of p53 in tumours emerge as a promising approach for the treatment of some cancer patients (Zuckerman *et al.*, 2009).

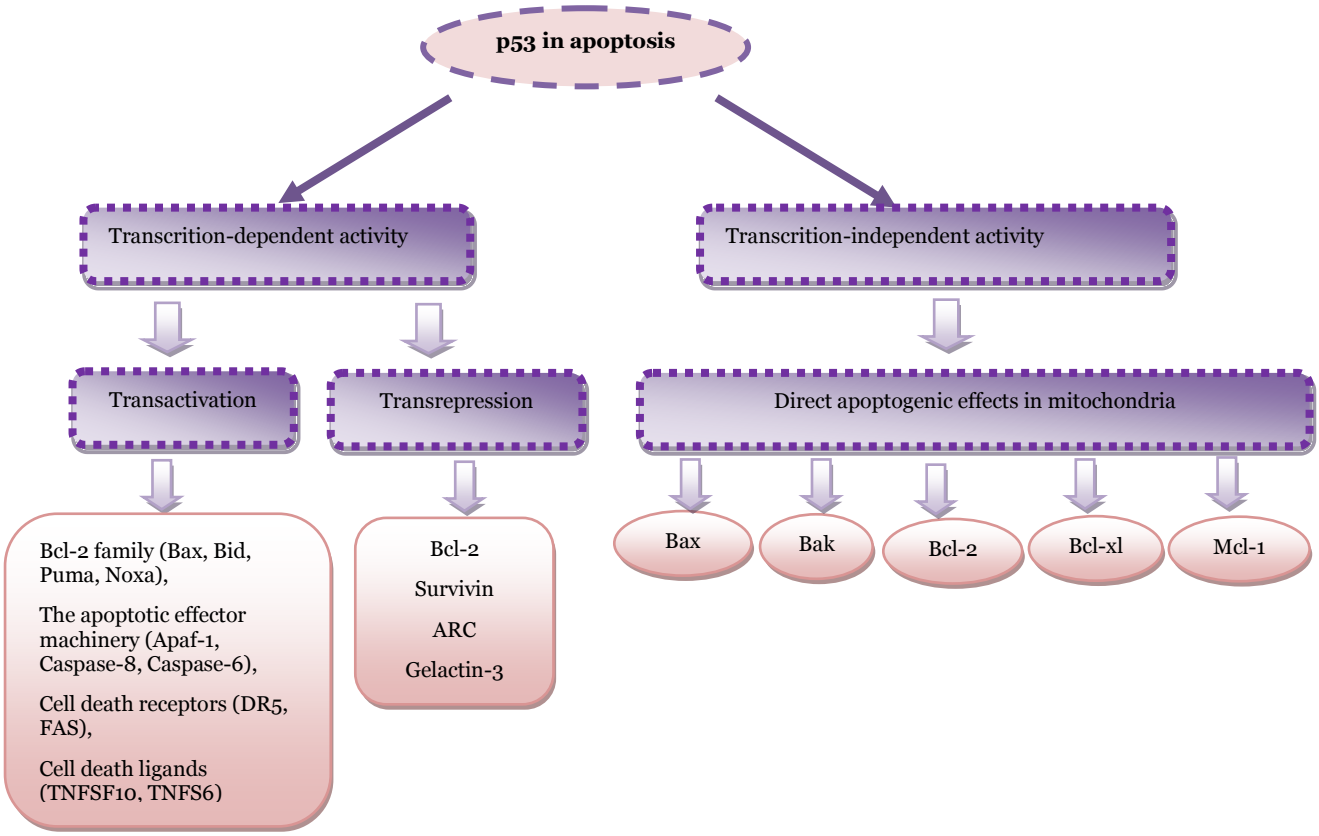


Figure 10. Multiple mechanisms involved in p53-mediated apoptosis [adapted from (Zuckerman et al., 2009)].

A possible approach for the treatment of cancer may be targeting cell cycle (De Falco and De Luca, 2010) and/or apoptosis (Ferreira *et al.*, 2010) pathways. But much remains to be done in the pursuit of agents capable of targeting such cellular process (Senderowicz, 2004).

4. MUSHROOMS WITH PROMISING ANTIOXIDANT AND TUMOUR CELL GROWTH INHIBITORY ACTIVITIES

When considering natural species as sources of compounds with potential medicinal properties, including antioxidant and tumour cell growth inhibitory activities, mushrooms appear represent important sources of therapeutically useful biologically active agents (Mizuno, 1995; Wasser, 1995; Wasser, 2002; Ferreira *et al.*, 2010).

Wild mushrooms contain different antioxidants such as phenolic compounds, tocopherols, ascorbic acid and carotenoids, which could be extracted for the purpose of being used as functional ingredients, namely against chronic diseases related to oxidative stress (Ferreira *et al.*, 2009). Different wild mushroom species have been reported to have antioxidant activity, as described in Table 2.

Table 2. Studies on Antioxidant Properties of Wild Mushrooms [Adapted from (Ferreira *et al.*, 2009)].

Mushroom species	Antioxidant Activity Assays	Country
Agaricus arvensis	DPPH scavenging activity; Hemolysis inhibition. Reducing power; TBARS assay; β-carotene bleaching inhibition;	Portugal
Agaricus bisporus	ABTS+ radical cation scavenging activity; Chelating effects on cupric ions; Chelating effects on ferrous ions; DPPH scavenging activity; FAD assay; Hemolysis inhibition; Hydroxyl radicals scavenging activity; Inhibition of lipid peroxidation; Linoleic acid assay; Reducing power; Superoxide anion radical scavenging activity; TBARS assay; Thiocyanate method. β-carotene bleaching inhibition;	China Korea Portugal Serbia Spain Turkey
Agaricus bitorquis	ABTS+ radical cation scavenging activity; Chelating effects on cupric ions; Chelating effects on ferrous ions; DPPH scavenging activity; β-carotene bleaching inhibition.	Turkey
Agaricus blazei	$ABTS^{+}$ radical cation scavenging activity. Chelating effects on ferrous ions; Conjugated diene method; DCF/AAPH assay; DPPH scavenging activity; Hydroxyl radicals scavenging activity; Hydroxyl radicals scavenging activity; Reducing power; β-carotene bleaching inhibition.	Brazil Korea Taiwan
Agaricus brasiliensis	Chelating ability on ferrous ions; DPPH scavenging activity; FAD assay; Inhibition of lipid peroxidation.	Serbia
Agaricus campestris	DPPH scavenging activity; Reducing power;	Portugal

Mushroom species	Antioxidant Activity Assays	Country
	β-carotene bleaching inhibition.	
Agaricus comtulus	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Agaricus essettei	ABTS+ radical cation scavenging activity; Chelating effects on cupric ions; Chelating effects on ferrous ions; DPPH scavenging activity; β-carotene bleaching inhibition.	Turkey
Agaricus lutosus	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Agaricus romagnesii	DPPH scavenging activity; Hemolysis inhibition. Reducing power; TBARS assay; β -carotene bleaching inhibition.	Portugal
Agaricus silvaticus	DPPH scavenging activity; Hemolysis inhibition; Reducing power; TBARS assay; β-carotene bleaching inhibition.	Portugal
Agaricus silvicola	DPPH scavenging activity; Hemolysis inhibition. Reducing power; TBARS assay; β-carotene bleaching inhibition.	Portugal
Agrocybe aegerita	$ABTS^{+}$ radical cation scavenging activity. DPPH scavenging activity; TBARS assay;	China
Agrocybe cylindracea	Chelating effects on ferrous ions; Conjugated diene method; DETBA method; DPPH scavenging activity; Hydroxyl radicals scavenging activity; Linoleic acid assay Reducing power.	Spain Taiwan
Amanita caesarea	Chelating effects on ferrous ions; DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Turkey
Amanita rubescens	DPPH scavenging activity.	Portugal
Amanita umbrinolutea	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Armillariella mellea	TBARS assay.	Taiwan
Auricularia aurícula	TBARS assay; Hydroxyl radicals scavenging activity	India
Auricularia polytricha	DPPH scavenging activity; Reducing power; TBARS assay.	India
Boletus armeniacus	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Boletus badius	Superoxide anion radical scavenging activity; Reducing power; DPPH scavenging activity; Chelating effects on ferrous ions; Thiocyanate method.	Turkey
Boletus edulis	Chelating effects on ferrous ions. Conjugated diene method;	India Portugal

Mushroom species	Antioxidant Activity Assays	Country
	DPPH scavenging activity; Hydroxyl radicals scavenging activity; Linoleic acid assay. Reducing power; TBARS assay; β-carotene bleaching inhibition.	Taiwan Spain Turkey
Boletus erythropus	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Boletus fragrans	DPPH scavenging activity; Reducing power. β-carotene bleaching inhibition;	Portugal
Boletus impolitus	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Bovista aestivalis	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Bovista nigrescens	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Calocybe gambosa	Linoleic acid assay.	Spain
Calvatia utriformis	β-carotene bleaching inhibition; DPPH scavenging activity; Reducing power.	Portugal
Cantharellus cibarius	DPPH scavenging activity; FRAP assay; Ferrous ion-chelating effect; Linoleic acid assay. Reducing power; TBARS assay; β-carotene bleaching inhibition.	Turkey India Portugal Spain
Cantharellus clavatus	DPPH scavenging activity; Reducing power; TBARS assay.	India
Cantharellus lutescens	Chelating effects on ferrous ions; DETBA method; DPPH scavenging activity; Hydroxyl radicals scavenging activity; Reducing power.	Spain
Catathelasma ventricosum	DPPH scavenging activity; Reducing power; Metal chelating activity.	China
Chlorophyllum rhacodes	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Clavariadelphus pistillaris	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Clavariadelphus truncatus	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Clitocybe alexandri	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Clitocybe costata	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Clitocybe geotropa	Chelating effects on ferrous ions; DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Turkey

Mushroom species	Antioxidant Activity Assays	Country
Clitocybe gibba	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Clitocybe maxima	DPPH scavenging activity; Metal chelating activity; Reducing power.	China
Clitopilus prunulus	β-carotene bleaching inhibition; DPPH scavenging activity; Reducing power.	Portugal
Coriolus versicolor	Chelating effects on ferrous ions DETBA method; DPPH scavenging activity. Hydroxyl radicals scavenging activity; Reducing power.	Taiwan
Cortinarius glaucopus	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Cortinarius praestans	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Craterellus cornucopioides	DPPH scavenging activity; Linoleic acid assay; Metal chelating activity; Reducing power.	China Spain
Dictophora indusiata	Chelating effects on ferrous ions DETBA method; DPPH scavenging activity. Hydroxyl radicals scavenging activity; Reducing power.	Taiwan
Fistulina hepatica	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Flammulina velutipes	Chelating effects on ferrous ions DETBA method; DPPH scavenging activity; Hydroxyl radicals scavenging activity; Reducing power; β-carotene bleaching inhibition.	Korea Portugal Taiwan
Ganoderma applanatum	Chelating ability on ferrous ions; DPPH scavenging activity; Ferric reducing antioxidant power assay; Hydroxyl radicals scavenging activity. Inhibition of lipid peroxidation; TBARS assay.	India Serbia
Ganoderma lucidum	Chelating ability on ferrous ions; DETBA method; DPPH scavenging activity. Ferric reducing antioxidant power assay; Hydroxyl radicals scavenging activity; Inhibition of lipid peroxidation; Reducing power; β-carotene bleaching inhibition.	Korea Portugal Serbia Taiwan
Ganoderma tsugae	DETBA method; Reducing power; DPPH scavenging activity; Hydroxyl radicals scavenging activity; Chelating effects on ferrous ions	Taiwan
Geastrum arinarius	DPPH scavenging activity; Reducing power; TBARS assay.	India
Geastrum saccatum	Superoxide anion radical scavenging activity; TBARS assay; Hydroxyl radicals scavenging activity	Brazil

Mushroom species	Antioxidant Activity Assays	Country
Grifola frondosa	Chelating effects on ferrous ions; Conjugated diene method. DETBA method; DPPH scavenging activity; Hydroxyl radicals scavenging activity; Reducing power; SOD activity.	Korea Taiwan
Helvella crispa	Chelating effects on ferrous ions; DPPH scavenging activity; Hydrogen peroxide scavenging activity. Reducing power; Superoxide anion radical scavenging activity; TBARS assay.	India Turkey
Hericium erinaceus	Chelating effects on ferrous ions; DETBA method; DPPH scavenging activity. Hydroxyl radicals scavenging activity; Reducing power.	Taiwan
Hydnum repandum	Chelating effects on ferrous ions; DETBA method; DPPH scavenging activity; Hydroxyl radicals scavenging activity; Linoleic acid assay Reducing power; TBARS assay. β-carotene bleaching inhibition.	India Portugal Spain
Hygrophoropsis aurantiaca	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Hygrophorus agathosmus	DPPH scavenging activity.	Portugal
Hygrophorus chrysodon	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Hygrophorus marzuolus	Linoleic acid assay.	Spain
Hygrophorus pustulatus	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Hypholoma capnoides	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Hypholoma fasciculare	DPPH scavenging activity; Reducing power; TBARS assay; β-carotene bleaching inhibition.	Portugal
Hypsizigus marmoreus	Reducing power; DPPH scavenging activity; Hydroxyl radicals scavenging activity; Conjugated diene method; Chelating effects on cupric ions.	Taiwan
Inonotus obliquus	Superoxide anion radical scavenging activity; DPPH scavenging activity; DCF/AAPH assay.	Korea
Laccaria amethystea	DPPH scavenging activity; Metal chelating activity; Reducing power.	China Portugal
Laccaria laccata	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Lactarius aurantiacus	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal

Mushroom species	Antioxidant Activity Assays	Country
Lactarius deliciosus	DPPH scavenging activity; FRAP assay; Ferrous ion-chelating effect; Hemolysis inhibition. Linoleic acid assay. Reducing power; TBARS assay. β-carotene bleaching inhibition.	India Portugal Spain Turkey
Lactarius deterrimus	Reducing power; DPPH scavenging activity; Chelating effects on ferrous ions; β-carotene bleaching inhibition.	Turkey
Lactarius piperatus	DPPH scavenging activity; Hemolysis inhibition. Reducing power; Superoxide anion radical scavenging activity; β-carotene bleaching inhibition.	Portugal
Lactarius salmonicolor	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Lactarius sanguifluus	Chelating effects on ferrous ions; DPPH scavenging activity. Hydrogen peroxide scavenging activity; Reducing power; Superoxide anion radical scavenging activity; TBARS assay.	India Turkey
Laetiporus sulphureus	DPPH scavenging activity; β-carotene bleaching inhibition.	Turkey
Lentinula edodes	$ABTS^{+}$ radical cation scavenging activity. Chelating ability on ferrous ions; DETBA method; DPPH scavenging activity; Ferric reducing antioxidant power assay; Hemolysis inhibition; Hydroxyl radicals scavenging activity; Inhibition of lipid peroxidation; Linoleic acid assay Reducing power; TBARS assay; β-carotene bleaching inhibition.	Brazil China Korea Serbia Spain Taiwan
Lentinus squarrulosus	DPPH scavenging activity. Reducing power; TBARS assay;	India
Lentius sajor-caju	TBARS assay; Reducing power; DPPH scavenging activity.	India
Lepista inversa	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Lepista nuda	Chelating effects on ferrous ions; DETBA method; DPPH scavenging activity. Hydroxyl radicals scavenging activity; Linoleic acid assay Reducing power; Superoxide anion radical scavenging activity; TBARS assay; Thiocyanate method. β -carotene bleaching inhibition.	Portugal Spain Turkey
Lepista sordida	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Leucoagaricus leucothite	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal

Mushroom species	Antioxidant Activity Assays	Country
Leucoagaricus pudicus	Chelating effects on ferrous ions; DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Turkey
Leucopaxillus giganteus	DPPH scavenging activity; Hemolysis inhibition. Hemolysis inhibition; Reducing power; β-carotene bleaching inhibition.	Portugal
Lycoperdon echinatum	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Lycoperdon molle	DPPH scavenging activity; Reducing power; TBARS assay; β-carotene bleaching inhibition.	Portugal
Lycoperdon perlatum	DPPH scavenging activity; Reducing power; TBARS assay; β-carotene bleaching inhibition.	Portugal
Lycoperdon umbrinum	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Lyophyllum decastes	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Macrolepiota excoriata	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Macrolepiota mastoidea	DPPH scavenging activity; Hemolysis inhibition; Reducing power; TBARS assay; β -carotene bleaching inhibition.	Portugal
Macrolepiota procera	DPPH scavenging activity. Hemolysis inhibition; Reducing power; TBARS assay; β-carotene bleaching inhibition.	India Portugal
Morchella angusticeps	DPPH scavenging activity; Reducing power; TBARS assay.	India
Morchella conica	Chelating effects on ferrous ions; DPPH scavenging activity. Hydrogen peroxide scavenging activity; Reducing power; Superoxide anion radical scavenging activity; TBARS assay.	India Turkey
Morchella esculanta	Chelating effects on ferrous ions; Conjugated diene method. DPPH scavenging activity; Hydrogen peroxide scavenging activity Reducing power; Superoxide anion radical scavenging activity.	Taiwan Turkey
Morchella vulgaris	Chelating effects on ferrous ions; DPPH scavenging activity; Hydrogen peroxide scavenging activity; Reducing power; Superoxide anion radical scavenging activity.	Turkey
Mycena rosea	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Phellinus linteus	CAM assay. Chelating ability on ferrous ions.	Korea Serbia

Mushroom species	Antioxidant Activity Assays	Country
	DPPH scavenging activity. FAD assay; Inhibition of lipid peroxidation; TBARS assay; Xanthine oxidase inhibition.	
Phellinus rimosus	Nitric oxide scavenging activity; Superoxide anion radical scavenging activity; TBARS assay.	India
Picoa juniperi	Chelating effects on ferrous ions; DETBA method; DPPH scavenging activity; Hydroxyl radicals scavenging activity; Linoleic acid assay; Reducing power.	Spain
Pleurotus citrinopileatus	DPPH scavenging activity. Reducing power.	Taiwan
Pleurotus cystidiosus	Chelating effects on ferrous ions; DETBA method; DPPH scavenging activity; Hydroxyl radicals scavenging activity; Reducing power.	Taiwan
Pleurotus djamor	DPPH scavenging activity; Reducing power; TBARS assay.	India
Pleurotus eryngii	DPPH scavenging activity.	Korea
Pleurotus ostreatus	Chelating effects on ferrous ions DETBA method; DPPH scavenging activity; Hydroxyl radicals scavenging activity; Linoleic acid assay; Reducing power; Superoxide anion radical scavenging activity; TBARS assay; Thiocyanate method.	India Korea Spain Taiwan Turkey
Pleurotus sajor-caju	DPPH scavenging activity; Reducing power; TBARS assay.	India
Polyporus annosus	DPPH scavenging activity; Ferrous ion-chelating effect; FRAP assay; β-carotene bleaching inhibition.	Turkey
Polyporus badius	DPPH scavenging activity; Ferrous ion-chelating effect; FRAP assay; β-carotene bleaching inhibition.	Turkey
Polyporus fomentarius	DPPH scavenging activity; Ferrous ion-chelating effect; FRAP assay; β-carotene bleaching inhibition.	Turkey
Polyporus gilvus	DPPH scavenging activity; Ferrous ion-chelating effect; FRAP assay; β-carotene bleaching inhibition.	Turkey
Polyporus pinicola	DPPH scavenging activity; Ferrous ion-chelating effect; FRAP assay; β-carotene bleaching inhibition.	Turkey
Polyporus radiatus	DPPH scavenging activity; Ferrous ion-chelating effect; FRAP assay; β-carotene bleaching inhibition.	Turkey
Polyporus squamosus	Chelating effects on ferrous ions; DPPH scavenging activity;	Turkey

Mushroom species	Antioxidant Activity Assays	Country
	Reducing power; Superoxide anion radical scavenging activity; Thiocyanate method.	
Polyporus stevenii	DPPH scavenging activity; Ferrous ion-chelating effect; FRAP assay; β-carotene bleaching inhibition.	Turkey
Polyporus sulphureus	DPPH scavenging activity; Ferrous ion-chelating effect; FRAP assay; β-carotene bleaching inhibition.	Turkey
Polyporus volvatus	DPPH scavenging activity; Ferrous ion-chelating effect; FRAP assay; β-carotene bleaching inhibition.	Turkey
Ramaria aurea	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Ramaria botrytis	DPPH scavenging activity; Reducing power; TBARS assay; β-carotene bleaching inhibition.	Portugal
Russula brevepis	DPPH scavenging activity; Reducing power; TBARS assay.	India
Russula cyanoxantha	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Russula delica	Chelating effects on ferrous ions; DPPH scavenging activity; Reducing power; Superoxide anion radical scavenging activity; Thiocyanate method; β-carotene bleaching inhibition.	Portugal Turkey
Russula olivacea	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Russula vesca	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Sarcodon imbricatus	DPPH scavenging activity; Hemolysis inhibition. Reducing power; TBARS assay; β-carotene bleaching inhibition.	Portugal
Sparassis crispa	DPPH scavenging activity; Reducing power; TBARS assay.	India Korea
Stropharia rugoso-annulata	DPPH scavenging activity; Metal chelating activity; Reducing power.	China
Suillus granulatus	DPPH scavenging activity.	Portugal
Suillus bellini	DPPH scavenging activity.	Portugal
Suillus collinitus	Chelating effects on ferrous ions; DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal Turkey
Suillus granulatus	DPPH scavenging activity.	Portugal
Suillus luteus	DPPH scavenging activity.	Portugal

Mushroom species	Antioxidant Activity Assays	Country
Suillus mediterraneensis	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Suillus variegatus	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Terfezia claveryi	Chelating effects on ferrous ions; DETBA method; DPPH scavenging activity. Hydroxyl radicals scavenging activity; Linoleic acid assay Reducing power;	Spain
Termitomyces albuminosus	Chelating effects on ferrous ions; Conjugated diene method; DPPH scavenging activity; Hydroxyl radicals scavenging activity; Reducing power.	Taiwan
Termitomyces heimii	Chelating effects on ferrous ions; DPPH scavenging activity; Hydrogen peroxide scavenging activity. Reducing power; Superoxide anion radical scavenging activity; TBARS assay.	India Turkey
Termitomyces microcarpus	DPPH scavenging activity; Reducing power; TBARS assay.	India
Termitomyces mummiformis	DPPH scavenging activity; Reducing power; TBARS assay.	India
Termitomyces shimperi	DPPH scavenging activity; Reducing power; TBARS assay.	India
Termitomyces tylerance	Chelating effects on ferrous ions; DPPH scavenging activity. Hydrogen peroxide scavenging activity. Reducing power; Superoxide anion radical scavenging activity; TBARS assay.	India Turkey
Trametes versicolor	Chelating ability on ferrous ions; DPPH radical scavenging activity; FRAP assay; Ferrous ion-chelating effect; Inhibition of lipid peroxidation; β-carotene bleaching inhibition.	Serbia Turkey
Trichloma giganteum	Chelating effects on ferrous ions DETBA method; DPPH scavenging activity; Hydroxyl radicals scavenging activity; Reducing power.	Taiwan
Tricholoma acerbum	DPPH scavenging activity; Reducing power; TBARS assay; β-carotene bleaching inhibition.	Portugal
Tricholoma equestre	DPPH scavenging activity.	Portugal
Tricholoma sulphureum	DPPH scavenging activity; Reducing power; β-carotene bleaching inhibition.	Portugal
Tricholomopsis rutilans	DPPH scavenging activity.	Portugal
Verpa conica	Superoxide anion radical scavenging activity; Reducing power; DPPH scavenging activity; Chelating effects on ferrous ions;	Turkey

Mushroom species	Antioxidant Activity Assays	Country
	Thiocyanate method.	
Volvariella volvacea	DPPH scavenging activity: Hemolysis inhibition; TBARS assay. β-carotene bleaching inhibition.	China
Xerocomus chrysenteron	Reducing power; DPPH scavenging activity; Chelating effects on ferrous ions; β-carotene bleaching inhibition.	Turkey

This table was elaborated based on the information available in the review article (Ferreira et al. 2009) and the following references on the same topic published since 2009: Heleno et al., 2010; Sarikurkcu et al., 2010; Grangeia et al., 2011; Öztürk et al., 2011; Orhan and Üstün, 2011; Palacios et al., 2011; Kozarski et al., 2012; Liu et al., 2012; Pereira et al., 2012; Tiana et al., 2012.

Over the last decades, natural compounds have drawn a great deal of attention from both the scientific community and the general public, as cancer chemopreventive agents and also as cancer therapeutics (Nobili *et al.*, 2009).

Mushrooms comprise a vast and yet largely untapped source of powerful new pharmaceutical products. In particular, and most importantly for modern medicine, they represent an unlimited source of compounds which are modulators of tumour cell growth. Furthermore, they may have potential as functional foods and sources of novel molecules.

The compounds with antitumour potential identified so far in mushrooms, can be classified according to their molecular weight these will include low-molecular-weight compounds (LMW, e.g. quinones, cerebrosides, isoflavones, catechols, amines, triacylglycerols, sesquiterpenes, steroids, organic germanium and selenium) and high-molecular-weight compounds (HMW, e.g. homo and heteroglucans, glycans, glycoproteins, glycopeptides, proteoglycans, proteins and RNA-protein complexes) (Figure 11).

These compounds have been reported to prevent tumourigenesis and also to suppress the growth of established tumours (Fulda, 2010). Particularly, mushrooms with potentially useful properties against cancers of the stomach, esophagus, lungs, etc. are known in China, Japan, Korea, Russia, United States and Canada (Ferreira *et al.*, 2010). Tables 3 and 4, and Figure 11 describe different types of natural compounds isolated from wild mushrooms with antitumoural potential.

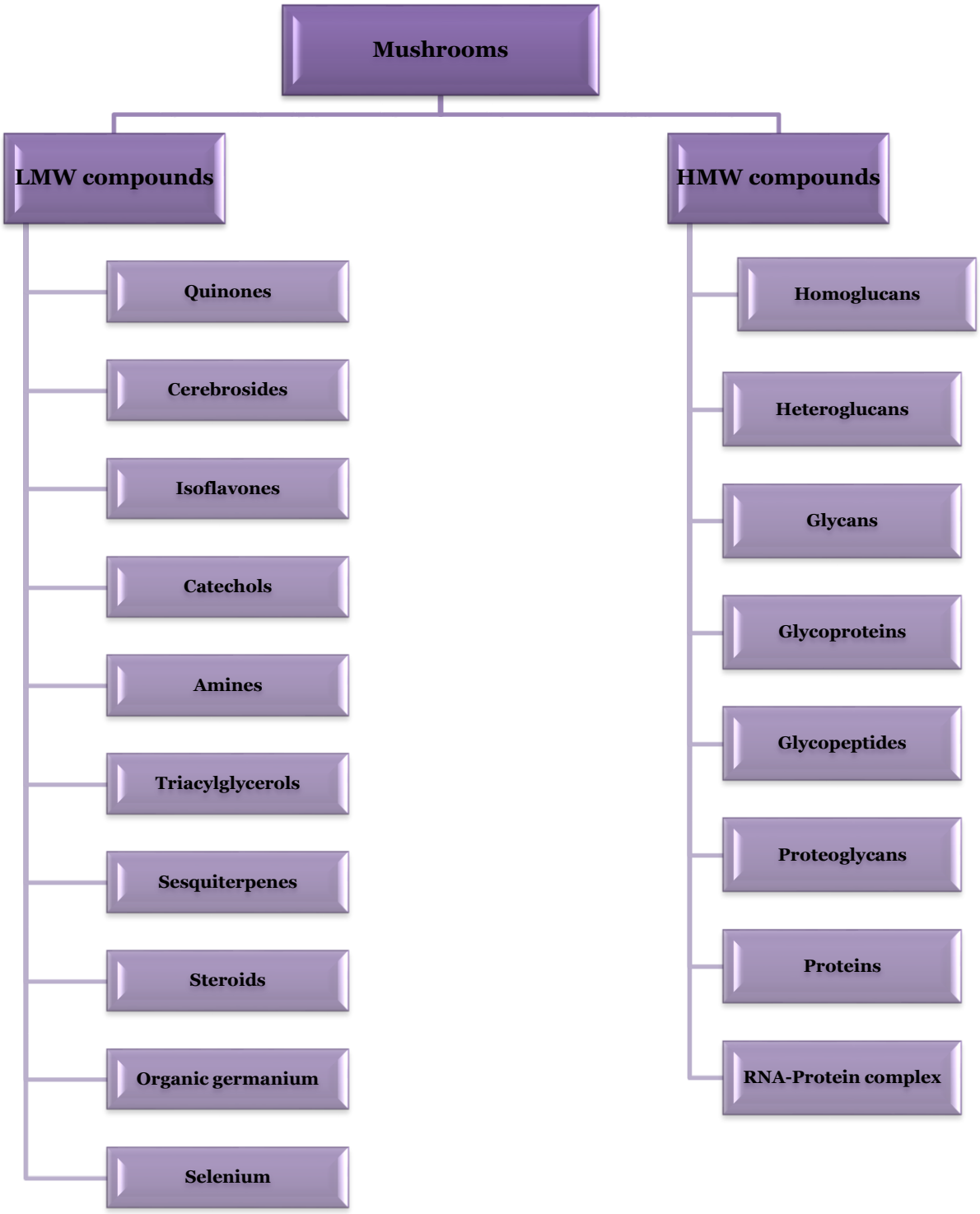


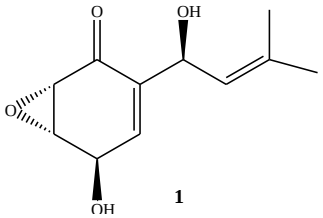
Figure 11. Low-molecular-weight (LMW) and high-molecular-weight (HMW) compounds with antitumour potential found in mushrooms [adapted from (Ferreira *et al*., 2010)].

Table 3. Studies on low-molecular-weight compounds with antitumour potential found in wild mushrooms [Adapted from (Ferreira *et al.*, 2010)].

Mushroom species	Antitumour agents	Molecular targets
Agaricus bisporus	490 Quinone (γ-L-glutaminyl-4-hydroxy-2,5-benzoquinone) (**4**)	DNA polymerase α inhibitor
	Selenium	DNA cytosine methyltransferase inhibitor
Agaricus blazei	Ergosterol (**15**)	Cyclooxygenase inhibitor and anti-angiogenic
Boletus edulis	Selenium	DNA cytosine methyltransferase inhibitor
Clitocybe clavipes	Clavilactones CB (**3a**), CD (**3b**) and CA (**3c**)	Tyrosine kinase inhibitors
Cordyceps sinensis	5,8-Epidioxy-24(*R*)-methylcholesta-6,22-dien-3β-ol (**14a**)	Sulfatase inhibitor
	5. 5,8-Epidioxy-24(*R*)-methylcholesta-6,22-dien-3β-D-glucopyranoside (**14b**) 6. 5,6-Epoxy-24(*R*)-methylcholesta-7,22-dien-3β-ol (**14c**)	Not known
Daedalea dickinsii	Polyporenic acid C (**24**)	MMPs inhibitor
Flammulina velupites	Genistein (**7**)	Cdc2 kinase modulator
	Selenium	DNA cytosine methyltransferase inhibitor
Fomitella fraxinea	Fomitellic acids A and B (**26a,b**)	DNA polymerase α and β inhibitors
Ganoderma applanatum	Ergosta-4,6,8(14),22-tetraen-3-one (**16**)	Cyclooxygenase inhibitor
Ganoderma lucidum	(4E,8E)-*N*-D-2'-hydroxypalmitoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine (**6a**)	DNA polymerase α inhibitors
	(4E,8E)-*N*-D-2'-hydroxystearoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine (**6b**)	DNA polymerase α inhibitors
	Lucidenic acid O (**17a**) Lucidenic lactone (**17b**)	DNA polymerase α, β and RT inhibitors (17a and 17b)
	Cerevisterol (**18**)	DNA polymerase α inhibitor (18)
	Lucidumol A (**19**) and B (**20a**) Ganoderiol F (**20b**)	NF-KB and AP-1 inhibitors (21a and 21c)
	Ganodermanondiol (**20c**) Ganodermanontriol (**20d**)	DNA topoisomerase inhibitor (23a)
	Ganoderic acids A (**21a**), F (**21b**), H (**21c**), W (**22**), X (**23a**), Y (**23b**), T (**23c**)	
	Bis-β-carboxyethylgermanium sesquioxide: O3(GeCH2CH2COOH)2	Not known
Ganoderma neo-japonicum	Ergosta-4,6,8(14),22-tetraen-3-one (**16**)	Cyclooxygenase inhibitor
Gerronema	Gerronemins A-F (**9a-f**)	COX-2 inhibitors
Grifola frondosa	1-Oleoyl-2-linoleoyl-3-palmitoylglycerol (**12**)	Cyclooxygenase inhibitor
	Ergosterol (**15**)	Cyclooxygenase inhibitor and anti-angiogenic
	Ergosta-4,6,8(14),22-tetraen-3-one (**16**)	Cyclooxygenase inhibitor
Gymnopilus marginatus	6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone (Hispidin) (**8**)	PKCβ inhibitor
Gymnopilus parvisporus	6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone (Hispidin) (**8**)	PKCβ inhibitor
Gymnopilus patriae	6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone (Hispidin) (**8**)	PKCβ inhibitor
Ionotus hispidus	6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone (Hispidin) (**8**)	PKCβ inhibitor
Lampteromyces japonicus	Iludin S and M (**13a,b**) and derivatives	The derivatives are DNA-alkylating agents
Lentinus crinitus	Panepoxydone (**1**)	NF-KB inhibitor
Lepiota americana	2-aminophenoxazin-3-one (**10**)	Aromatase inhibitor
	5,8-Epidioxy-24(*R*)-methylcholesta-6,22-dien-3β-ol (**14a**)	Sulfatase inhibitor

Mushroom species	Antitumour agents	Molecular targets
Omphalotus illudens	Iludin S and M (**13a,b**) and derivatives	The derivatives are DNA-alkylating agents
Phellinu linteus	6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone (Hispidin) (**8**)	PKCβ inhibitor
Panus conchatus	Panepoxydone (**1**)	NF-KB inhibitor
Panus rudis	Panepoxydone (**1**)	NF-KB inhibitor
Pholiota spumosa	Putrescine-1,4-dicinnamide (**11**)	Not known (inducer of apoptosis and necrosis)
Piptoporus betulinus	(E)-2-(4-hydroxy-3-methyl-2-butenyl)-hydroquinone (**5**)	MMPs inhibitor
Piptoporus betulinus	Polyporenic acid C (**24**)	MMPs inhibitor
Pleurotus ostreatus	Selenium	DNA cytosine methyltransferase inhibitor
Poria cocos	Dehydroebriconic acid (**25**)	DNA topoisomerase II inhibitor
***Xylaria* strain 45-93**	Cycloepoxydon (**2**)	NF-KB inhibitor

Quinones



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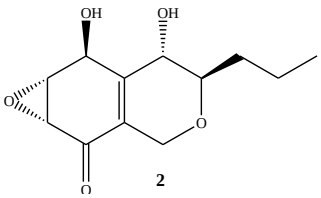

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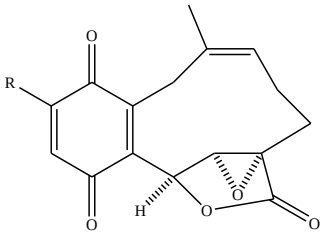
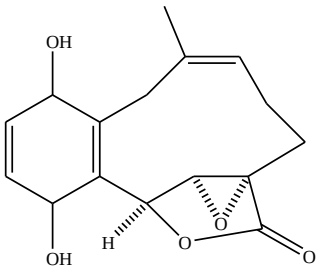
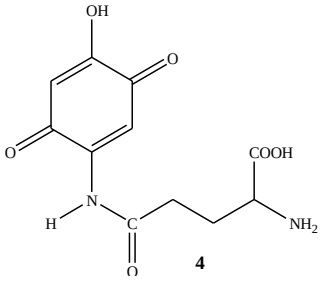


3a R=H; **3b** R=OH



3c



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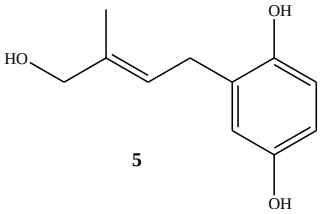

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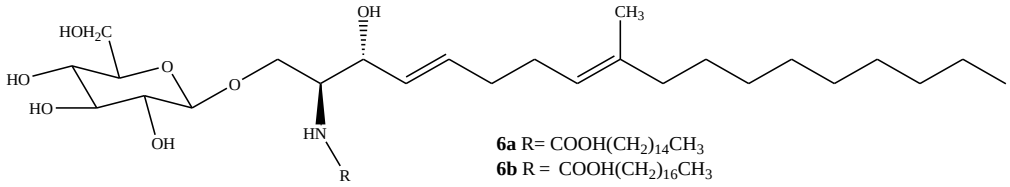
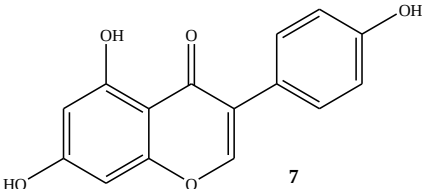
Cerebrosides



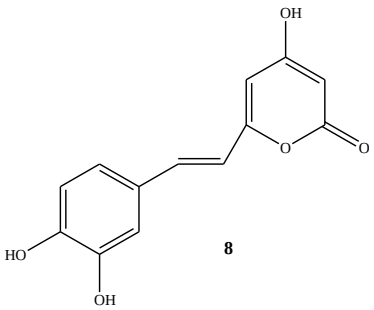
6a R= $COOH(CH_2)_{14}CH_3$
6b R = $COOH(CH_2)_{16}CH_3$

Isoflavones



7

Catechols



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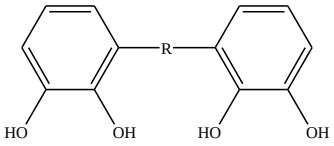
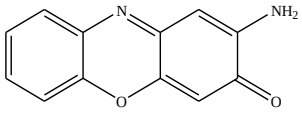


9a R= $(CH_2)_{12}$
9b R= $(CH_2)_3CH{=}CH(CH_2)_7$
9c R= $(CH_2)_{14}$
9d R= $(CH_2)_5CH{=}CH(CH_2)_7$
9e R= $CH_2CH{=}CH(CH_2)CH{=}CH(CH_2)_7$
9f R= $(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7$

Amines



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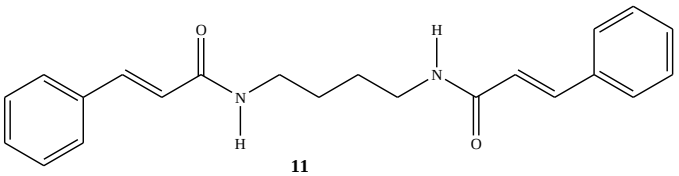
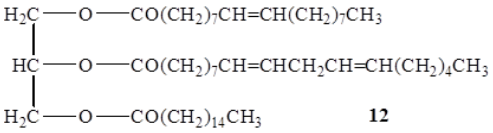


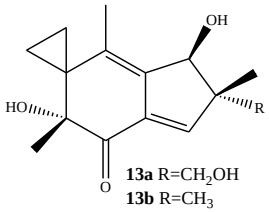
11

Triacylgycerols



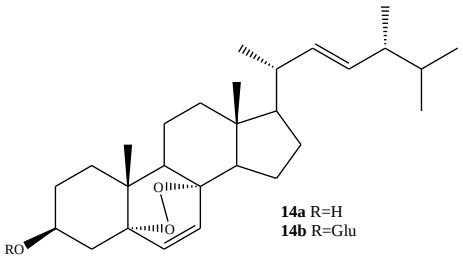
12

Sesquiterpenes

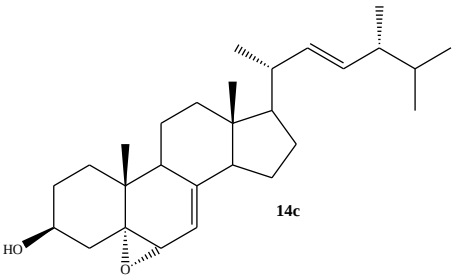


13a R=CH_2OH
13b R=CH_3

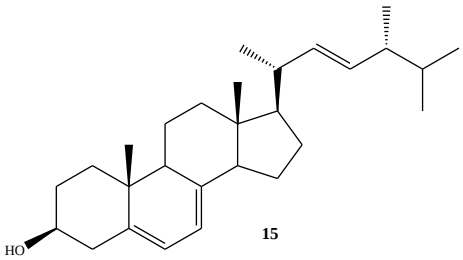
Steroids



14a R=H
14b R=Glu



14c



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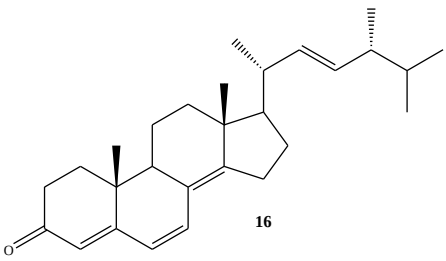

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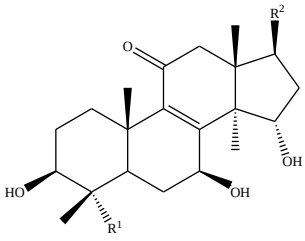
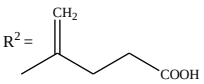
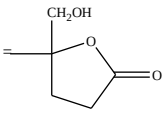


17a $R^1 = CH_2OH$, R^2 =



17b $R^1 = CH_3$, R^2 =



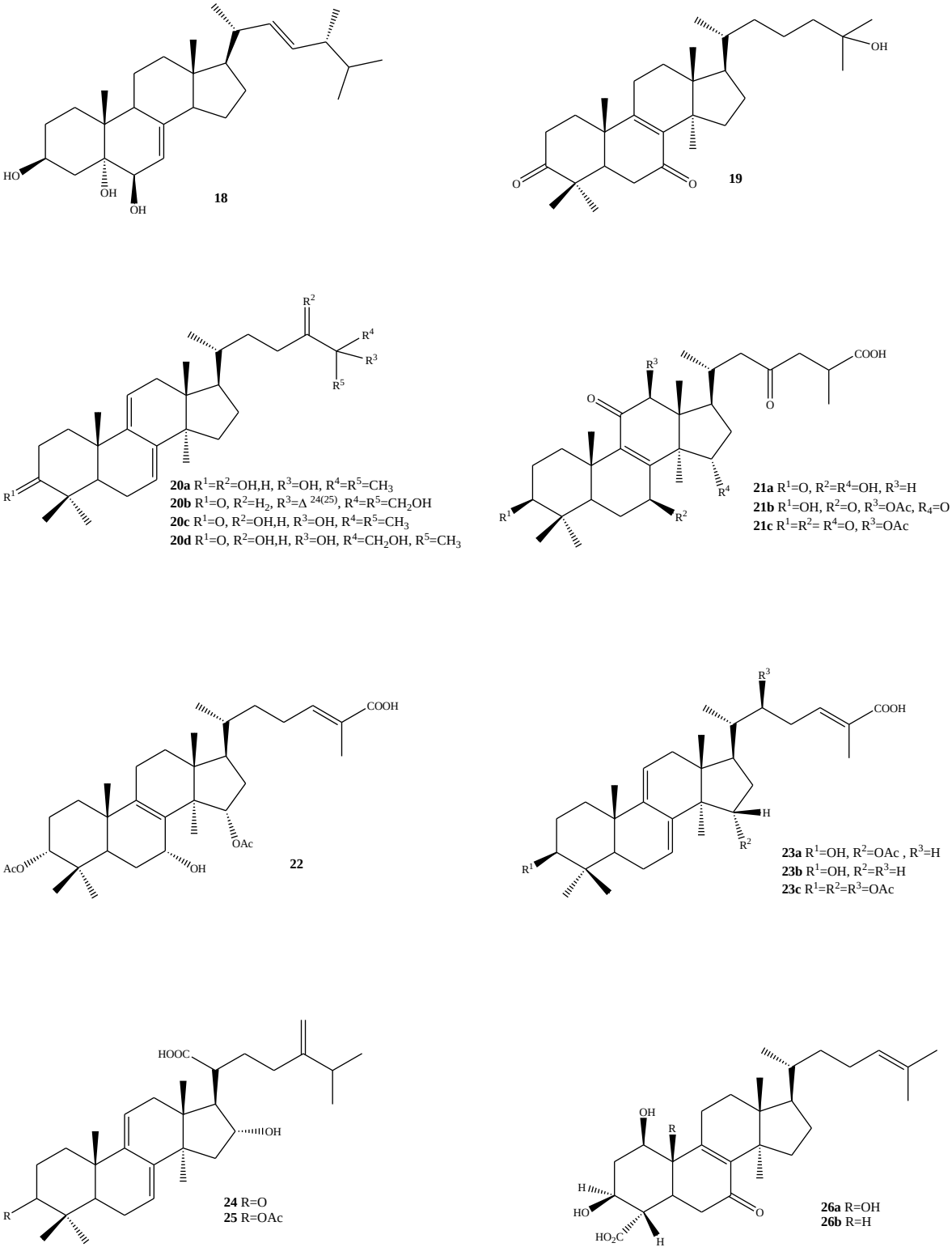


Figure 12. Chemical structure of the low-molecular-weight (LMW) compounds with antitumour potential found in mushrooms [Adapted from (Ferreira et al., 2010)].

Table 4. Studies on high-molecular-weight compounds with antitumour potential extracted of wild mushrooms [Adapted from (Ferreira *et al.*, 2010)].

Mushroom species	Antitumour agents	Details
Agaricus bisporus	Lectins	Immunomodulator and antiproliferative; *in vitro* cell lines
Agaricus blazei	ATOM	Immunomodulator*;* pre-clinical animal models
	AB-FP	Immunomodulator; clinical trials
	FA-2-b-Md	Immunomodulator*; in vitro* cell lines
	(1→6)-β-D-glucan with (1→3)-β-D branches:	Immunomodulator; clinical trials (breast, prostate, lung, liver, and gastric cancers)
	D-fraction	
	(1→6)-β-D-glucan with (1→4)- α branches	Immunomodulator; pre-clinical animal models
	(1→3)-α- glucan	Immunomodulator; pre-clinical animal models
	(1→3)-α–glucan with (1→6)-β branches	Immunomodulator; pre-clinical animal models
	(1→6)-α–glucan with (1→4)-α branches	Immunomodulator; pre-clinical animal models
	Mannogalactoglucan	Immunomodulator; *in vitro* cell lines
	Riboglucan	Immunomodulator; *in vitro* cell lines
	Xyloglucan	Immunomodulator; pre-clinical animal models
	Glucomannan	Immunomodulator; pre-clinical animal models
Agrocybe aegerita	(1→3)-α- glucan	Immunomodulator; pre-clinical animal models
Amanita muscaria	(1→3)-α- glucan	Immunomodulator; pre-clinical animal models
Armillariella tabescens	Linear (1→6)- β-D-glucan	Immunomodulator; pre-clinical animal models
	(1→3)-α- glucan	Immunomodulator; pre-clinical animal models
Auricularia auricula	Linear (1→3)-β-D-glucan	Immunomodulator; pre-clinical animal models
Boletus satanas	Lectins	Immunomodulator and antiproliferative; *in vitro* cell lines
Clitocybe nebularis	Clitocypin	Cysteine proteinase inhibitor; *in vitro* enzymatic inhibition assays
Cryptoporus volvatus	H-3-B	Immunomodulator; pre-clinical animal models
Dictyophora indusiata	Fucomannogalactan	Immunomodulator; pre-clinical animal models
Dictyophora indusiata	Mannan	Immunomodulator; pre-clinical animal models
Flammulina velutipes	Flammulin	Immunomodulator*; in vitro* cell lines
	Galactomannoglucan	Immunomodulator; pre-clinical animal models
	Riboglucan	Immunomodulator; *in vitro* cell lines
Fomitella fraxinea	Mannofucogalactan	Immunomodulator; *in vitro* cell lines
Ganoderma lucidum	Ganoderans	Immunomodulator*; in vitro* cell lines
	Glycopeptide complexes	Immunomodulator; pre-clinical animal models
	GLIS	Immunomodulator*;* pre-clinical animal models
	Protein LZ8	Immunomodulator*;* pre-clinical animal models
	GLP	Immunomodulator; pre-clinical animal models

Mushroom species	Antitumour agents	Details
	Mannogalactoglucan	Immunomodulator; *in vitro* cell lines
	(1→3)-β-glucuronoglucan	Immunomodulator; *in vitro* cell lines
Ganoderma tsugae	Arabinoglucan	Immunomodulator; pre-clinical animal models
	Glucogalactan	Immunomodulator; pre-clinical animal models
Grifola frondosa	Lectins	Immunomodulator and antiproliferative; *in vitro* cell lines
	Grifolan, GRN	Immuno-enhancing activity; clinical trials (gastrointestinal, lung, liver and breast cancers)
	(1→6)-β-D-glucan with (1→3)-β-D branches:	Immunomodulator; clinical trials (breast, prostate, lung, liver, and gastric cancers)
	D-fraction	
	Mannoxyloglucan	Immunomodulator; pre-clinical animal models
	Xyloglucan	Immunomodulator; pre-clinical animal models
	Fucomannogalactan	Immunomodulator; pre-clinical animal models
	Mannogalactofucan	Immunomodulator; pre-clinical animal models
Hericinum erinaceus	Galactoxyloglucan-protein complex	Immunomodulator*; in vitro* cell lines
	Glucoxylan	Immunomodulator; pre-clinical animal models
	Galactoxyloglucan	Immunomodulator; *in vitro* cell lines
	Xylan	Immunomodulator; pre-clinical animal models
	Mannoglucoxylan	Immunomodulator; pre-clinical animal models
Hericium caput-medusae	Glucoxylan-protein complex	Immunomodulator*; in vitro* cell lines
Hohenbuehelia serotina	Galactomannoglucan	Immunomodulator; pre-clinical animal models
Inonotus obluquus	Xylogalactoglucan	Immunomodulator; *in vitro* cell lines
Lentinus edodes	KS-2	Immunomodulator; *in vitro* cell lines
	LEM	Immunomodulator*;* pre-clinical animal models
	Lentinan	Immuno-enhancing activity; clinical trials (gastric, colorectal, prostate and breast cancers)
	Galactoglucomannan	Immunomodulator; pre-clinical animal models
Lentinus lepideus	PG101	Immunomodulator; pre-clinical animal models
Leucopaxillus giganteus	Galactomannoglucan	Immunomodulator; pre-clinical animal models
Lyophyllum decastes	Linear (1→3)-β-D-glucan	Immunomodulator; pre-clinical animal models
Lyophyllum decastes	Linear (1→6)- β-D-glucan	Immunomodulator; pre-clinical animal models
Morchella esculenta	Galactomannan	Immunomodulator; pre-clinical animal models
Pachyman* from *Poria cocos	(1→3)- β-D-glucan with (1→2) or (1→6) branches	Immunomodulator; pre-clinical animal models
Phellinus linteus	PL	Immunomodulator*;* pre-clinical animal models
Pleurotus citrinopileatus	Arabinogalactan	Immunomodulator; pre-clinical animal models
Pleurotus cornucopiae	Mannogalactoglucan	Immunomodulator; *in vitro* cell lines
Pleurotus ostreatus	Pleuran	Immunomodulator; pre-clinical animal models
Pleurotus pulmonariu	Mannogalactoglucan	Immunomodulator; *in vitro* cell lines
	Xyloglucan	Immunomodulator; pre-clinical animal models
	Mannogalactan	Immunomodulator; *in vitro* cell lines

Mushroom species	Antitumour agents	Details
	Glucoxylan	Immunomodulator; pre-clinical animal models
Pleurotus tuber-regium	Alkali-soluble glucan	Immunomodulator; *in vitro* cell lines
Polyporus confluens	Xyloglucan	Immunomodulator; pre-clinical animal models
Sarcodon aspratus	Fucogalactan	Immunomodulator; pre-clinical animal models
Schizophyllum commune	Schizophyllan, SPG	Immuno-enhancing activity; clinical trials (gastric, cervical, head and neck cancers)
Sclerotinia sclerotiorum	Scleroglucan, SSG	Immunomodulator; clinical trials
Sparassis crispa	SCG	Immunomodulator; pre-clinical animal models
Trametes versicolor	PSP	Immuno-enhancing activity; clinical trials
	PSK or krestin	Immuno-enhancing activity; clinical trials (head, neck, upper gastro-intestinal, colorectal, lung , and breast cancers)
Tremella fuciformis	Tremellastin (Glucuronoxylomannans)	Immunomodulator; pre-clinical animal models
Tricholoma lobayense	PSPC	Immunomodulator; pre-clinical animal models
Tricholoma mongolicum	Lectins	Immunomodulator and antiproliferative; *in vitro* cell lines
Volvariella volvacea	Lectins	Immunomodulator and antiproliferative; *in vitro* cell lines

As can be seen in Tables 2, 3 and 4, there are several compounds that might contain functional or medicinal properties, which may be used as a source of biologically and physiologically active substances. Therefore, it is very important to carry out studies concerning the isolation, structural characterization and classification of compounds from mushrooms presenting potential antioxidant and antitumoural properties.

5. FRAMEWORK AND OBJECTIVES

As previously stated, mushrooms represent a rich source of biologically active compounds with recognized potential in drug discovery and development (Harvey, 2000; Newman *et al.*, 2000). Mushrooms have been widely used in traditional oriental medicine and are a source of compounds with antitumour properties (Ferreira *et al.*, 2010).The most important are high-molecular-weight compounds (e.g. polysaccharides, glycoproteins, proteoglycans and proteins). Some examples are Krestin (PSK), from the cultured mycelium of *Coriolus versicolor*, Lentinan from the fruiting bodies of *Lentinus edodes* and Schizophyllan from the culture fluid of *Schizophyllum commune*. Lentinan and Schizophyllan are pure β-glucans, whereas PSK is a protein bound polysaccharide.

The biological activity of these three products is related to their immunomodulating properties, which enhance the body's defences against various forms of infectious disease (Mizuno, 1993; Zaidman *et al.*, 2005), and are being used along with chemotherapy and radiotherapy in China, Japan and Korea (Larone, 2002). Furthermore, the anti- prostate cancer stem cells effect of PSP (other active component extracted from C. versicolor) was demonstrated as being also the chemopreventive property of oral PSP consumption against prostate cancer (Luk *et al.*,. 2011).

Mushrooms are also rich sources of antioxidant compounds, such as phenolic compounds (mainly phenolic acids), followed by tocopherols, ascorbic acid and carotenoids (Ferreira *et al.*, 2009).

Nevertheless, there are many undiscovered mushrooms with possible antioxidant properties and tumour cell growth inhibitory activity. The Northeast of Portugal is, due to climatic conditions, one of the European regions with higher wild mushrooms diversity, some of them with great gastronomic relevance. Mushrooms collection and consumption is a traditional practice in this region. In most cases, mushrooms collection is for self-consumption, despite the risks of this practice. Nevertheless, in the recent years the collection of wild mushrooms for commercialisation purposes became intensified, due to national and international interest in their nutritional and medicinal properties. Therefore, their potential as functional foods and as sources for the development of drugs and nutraceuticals should be explored.

Thus, the specific aims of this work were:

- Collection and taxonomic identification of species of wild mushrooms from the Northeast of Portugal with potential medicinal interest.
- Preparation of extracts (phenolic and polysaccharidic) from those species and evaluation of their bioactive properties:
    - antioxidant properties using different techniques (DPPH radical-scavenging activity, reducing power, inhibition of β-carotene bleaching) and
    - effect on the growth of various human tumour cell lines (NCI-H460 - lung cancer, MCF-7 – breast cancer, HCT-15 - colon cancer, AGS - gastric cancer). This panel of tumour cell lines has been chosen to represent cancers with higher incidence nowadays.

- Evaluation of the effects of the most active extracts on cell cycle profile and apoptosis.
- Identification and quantification of some of the compounds related to the mentioned bioactivities.

CHAPTER II.

METHODOLOGY

1. WORKING PLAN

The working plan of this thesis is schematized in Figure 16 and was the following:

1) Collection and identification of mushroom samples. The taxonomic identification of sporocarps was made according to the recommendations of several authors (Marchand, 1971; Marchand, 1986; Bon, 1988; Courtecuisse and Duhem, 2005).

2) Extraction of the samples in order to obtain phenolic (rich in low molecular weight compounds) and polysaccharidic (rich in high molecular weight compounds) extracts. The samples were submitted to solid-liquid extractions using different solvents (methanol, ethanol and boiling water).

3) Chemical characterization of the extracts:
    a. phenolic compounds were analysed by high performance liquid chromatography coupled to a photodiode array detector (HPLC-DAD);
    b. sugars were determined, after polysaccharides hydrolysis, by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) using raffinose or fructose as internal standards (IS);
    c. the nutritional value of some species (when not previously reported in literature) was also evaluated.

4) Evaluation of antioxidant potential:
    a. *DPPH radical-scavenging activity assay* – DPPH is a radical of nitrogen which is synthetic and highly stable because of the delocalization of the free electron, responsible for the deep purple color characteristic of this molecule. DPPH reacts with compounds which are capable of donating a hydrogen atom and reducing it, with the formation of a pale yellow compound (hydrazine) (Antolovich *et al.* 2002; Amarowicz *et al.*, 2004);

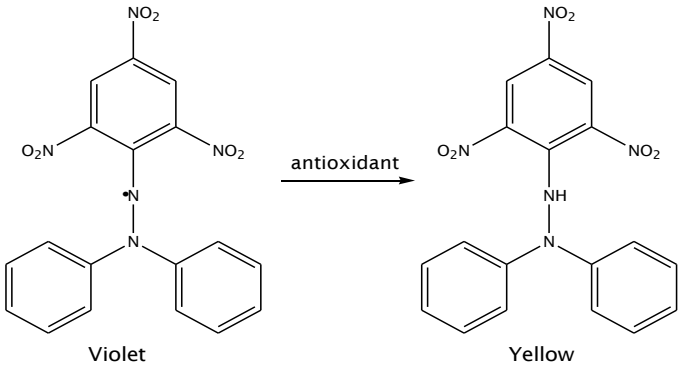


Figure 13. Reduction of DPPH radical (2,2-Diphenyl-1-picrylhydrazyl).

b. *Reducing power* assay- This assay measures the capacity of a given antioxidant reducing Fe(III)/ferricyanide [$FeCl_3/K_3Fe(CN)_6$] to Fe(II) (Berker, 2007). Thus, the yellow color of the solution is changed to Prussian blue, depending on the concentration of the antioxidant present in the solution. The reduction reaction takes place according to the following equations:

$$K_3FeCN_6 \xrightarrow{pH=6,6} 3K^+ + Fe(CN)_6^{3-}$$

$$\underbrace{Fe(CN)_6^{3-}}_{\text{Yellow}} + \text{antioxidant} \longrightarrow Fe(CN)_6^{4-} + \text{oxidated antioxidant}$$

$$Fe(CN)_6^{4-}\ Fe^{3+} \longrightarrow \underbrace{Fe[Fe(CN)_6]}_{\text{Prussian Blue}}$$

Figure 14. Reactions involved in the reducing power assay.

c. *Inhibition of β-carotene bleaching assay*. The antioxidant activity of the extracts was evaluated by the β-carotene linoleate model system (Mi-Yae *et al.*, 2003). This assay is based on spectrophotometric measurement of bleaching of the β-carotene, evaluating the scavenging activity on free radicals generated during the peroxidation of linoleic acid. The test is according to the following equations:

$$\beta - \text{carotene} - H(\text{laranja}) + ROO\cdot \longrightarrow \beta - \text{carotene}\cdot(\text{bleached}) + ROOH$$

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

Figure 15. Reactions involved in the inhibition of β-carotene bleaching assay.

This mechanism is involved in bleaching carotenoids by a thermal oxidation, which can be reduced by the action of the antioxidants in the sample (Amarowicz *et al.*, 2004; Kaur *et al.*, 2006). Thus, the decrease of the orange color of β-carotene is inversely proportional to the amount of antioxidants present in the extracts.

5) Screening for potential tumour cell growth inhibitors. The effects of the extracts on the growth of human tumour cell lines was evaluated, according to the procedure adopted in the NCI's *in vitro* anticancer drug screening, which uses the sulforhodamine B (SRB) assay to assess cell growth inhibition (Skehan *et al.*, 1990). This colorimetric assay estimates cell number indirectly, by staining cellular protein with the protein-binding dye SRB.

6) Selection of the most active extracts: ethanolic extract of *Clitocybe alexandri* in NCI-H460 cell line and methanolic extract of *Suillus collinitus* in MCF-7 cancer cell line.

7) Chemical characterization of compounds in the most active extracts.

8) Study of the effect of the most active extracts, and of their individual compounds, in the cell cycle distribution and in cellular apoptosis, by flow cytometry.

9) Finally, evaluation of the effect of the most active extracts on the levels of expression of some proteins involved in cell cycle and/or apoptosis (p53,p21, Bcl2, XIAP,PARP) by Western blotting.

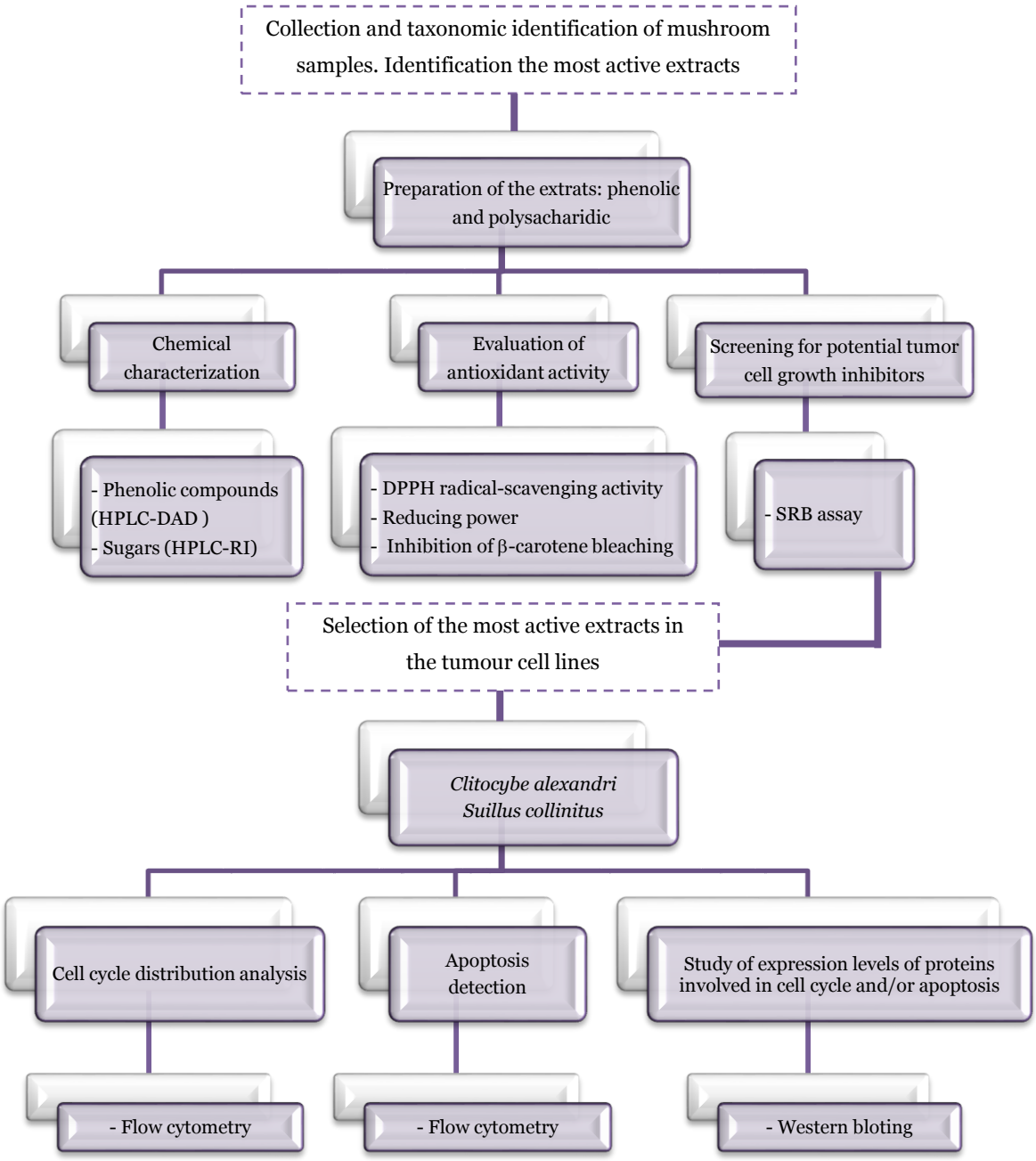


Figure 16. Schematic representation of the working plan.

2. EXPERIMENTAL

This section describes the author contributions to the technical procedures used in each experimental methodology which was conducted for this thesis. The experimental methods are described in the five papers (Annexes I - V) which form an integrated part of this thesis.

Collection and taxonomic identification of mushrooms from the Northeast of Portugal

All the samples were collected in different ecosystems of the Northeast of Portugal and taxonomically identified with the technical support of Prof. Anabela Martins, as described in the experimental part of Annexes I-V.

Preparation of mushrooms phenolic and polysaccharidic extracts

Phenolic and polysaccharidic extracts were obtained performing solid-liquid extractions with different solvents: methanol/ethanol and boiling water, respectively, at CIMO (Laboratory of Applied Chemistry and Biochemistry), as described in the experimental part of Annexes I - V.

Chemical characterization

The extracts were further characterized using High-Performance Liquid Chromatography (HPLC) coupled with different detectors: diode array (DAD) and refraction index (RI) detectors for phenolic compounds and sugars analysis, respectively, at CIMO (Laboratory of Applied Chemistry and Biochemistry), as described in the experimental part of Annexes I, II and V .

Antioxidant activity assays

The antioxidant activity assays (DPPH radical-scavenging activity, reducing power and inhibition of β-carotene bleaching) were performed at CIMO (Laboratory of Applied Chemistry and Biochemistry), as described in the experimental part of Annexes I, III and V.

Trypan blue exclusion assay

The Trypan blue exclusion assays were performed on four solid tumour cell lines: NCI-H460 (non-small cell lung cancer), MCF-7 (breast cancer), AGS (gastric cancer),

HCT-15 (colon cancer). This work was carried out at IPATIMUP (Cancer Drug Resistance Group), as described in the experimental part of Annex IV.

Sulphorhodamine B assay

The sulphorhodamine B (SRB) assay was performed on two leukemia cell lines, K562 and HL-60, and on four solid tumour cell lines: NCI-H460 (non-small cell lung cancer), MCF-7 (breast cancer), AGS (gastric cancer), HCT-15 (colon cancer). This work was carried out at IPATIMUP (Cancer Drug Resistance Group), as described in the experimental part of Annexes III and V. Some of the analyses were conducted with the technical support of Dr. Gabriela Almeida, Catarina Tavares or Diana Ferreira.

Flow Cytometry

Analysis of the cell cycle profile and apoptosis levels were performed by flow cytometry, at IPATIMUP (Cancer Drug Resistance Group), as described in the experimental part of Annexes IV and V. Some of the assays were conducted with the technical support of Dr. Gabriela Almeida.

Western Blotting

Western blots were performed at IPATIMUP (Cancer Drug Resistance Group), as described in the experimental part of Annexes IV and V. Some of the assays were conducted with the technical support of Dr. Gabriela Almeida or Catarina Tavares.

CHAPTER III.

RESULTS AND DISCUSSION

1. SAMPLES COLLECTION, IDENTIFICATION AND NUTRITIONAL CHARACTERIZATION

Thirty five samples (Table 5) were collected in different ecosystems (*Quercus pyrenaica*, *Pinus pinaster, Pinus sylvestris Ait* and mixed stands) of the Northeast of Portugal in the period from autumn 2007 until spring 2012.

The morphological identification of the wild macrofungi was made according to macro and microscopic characteristics (Marchand, 1971; Marchand, 1986; Bon, 1988; Courtecuisse and Duhem, 2005; Moreno, 2005) and online keys (http://www.mycokey.com/).

Representative voucher specimens were deposited at the herbarium of Instituto Politécnico de Bragança. After taxonomic identification, the mushrooms were immediately lyophilized (Ly-8-FM-ULE, Snijders, Holland).

The nutritional composition of *Armillaria mellea, Calocybe gambosum, Clitocybe odora, Coprinus comatus, Hygrophorus agathosmus, Hygrophorus olivaceo-albus, Russula caerulea* and *Tricholoma atrosquamosum* was performed in this work (Annex 1), while the other species were previously studied (Table 5).

Table 5. Information about the wild edible mushroom species collected identified and analyzed.

Species	Kingdom	Division	Class	Order	Family	Genus	Reference	Ecology	Ref.
Agaricus arvensis	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Agaricaceae	Agaricus	L.:Fr. emend Karst.	Saprotrophic	Barros et al., 2007
Agaricus bisporus	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Agaricaceae	Agaricus	L.:Fr. emend Karst.	Saprotrophic	Barros et al., 2008a
Armillaria mellea	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Physalacriaceae	Armillaria	(Vahl) P. Kumm.	Parasitic	Determined herein (Annex 1)
Boletus edulis	Fungi	Basidiomycota	Agaricomycetes	Boletales	Boletaceae	Boletus	Bull. (1782)	Mycorrhizal	Barros et al., 2008a
Calocybe gambosum	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Lyophyllaceae	Calocybe	(Fr.) Donk	Mycorrhizal	Determined herein (Annex 1)
Cantharellus cibarius (comercial)	Fungi	Basidiomycota	Agaricomycetes	Cantharellales	Cantharellaceae	Cantharellus	Adans. ex Fr.	Mycorrhizal	Barros et al., 2008a
Canthrelus cibarius (Silvestre)	Fungi	Basidiomycota	Agaricomycetes	Cantharellales	Cantharellaceae	Cantharellus	Adans. ex Fr.	Mycorrhizal	Barros et al., 2008b
Clitocybe alexandri	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Clitocybe	(Gillet) Konrad	Saprotrophic	Heleno et al., 2010
Clitocybe odora	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Clitocybe	(Fr.) P. Kumm	Mycorrhizal	Determined herein (Annex 2)
Coprinus comatus	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Agaricaceae	Coprinus	(O.F.Müll.) Pers.	Saprotrophic	Determined herein (Annex 1)
Craterellus cornucopioides	Fungi	Basidiomycota	Agaricomycetes	Cantharellales	Cantharellaceae	Craterellus	(L.) Pers.	Saprotrophic	Barros et al., 2008a
Fistulina hepatica	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Fistulinaceae	Fistulina	(Schaeff.) With.	Parasitic	Heleno et al., 2009
Flammulina velutipes	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Phisalacriaceae	Flammulina	(Curt.: Fries) Singer	Saprotrophic	Reis et al., 2012
Hydnum repandum	Fungi	Basidiomycota	Agaricomycetes	Cantharellales	Hydnaceae	Hydnum	(L.: Fr.)	Mycorrhizal	Heleno et al., 2010
Hygrophorus aurantiaca	Fungi	Basidiomycota	Agaricomycetes	Boletales	Hygrophoropsidaceae	Hygrophoropsis	(Wulfen) Maire	Mycorrhizal	Heleno et al., 2010
Hygrophorus agathosmus	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Hygrophorus	(Fr.) Fr.	Mycorrhizal	Determined herein (Annex 2)
Hygrophorus olivaceo-albus	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Hygrophorus	Fr. (Fr.)	Mycorrhizal	Determined herein (Annex 2)

Laccaria amethystina	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Hydnangiaceae	Laccaria	(Huds.) Cooke	Mycorrhizal	Heleno et al., 2010
Laccaria laccata	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Hydnangiaceae	Laccaria	(Scop.) Cooke	Mycorrhizal	Heleno et al., 2009
Lactarius deliciosus	Fungi	Basidiomycota	Agaricomycetes	Russulales	Russulaceae	Lactarius	(L. ex Fr.) S.F.Gray	Mycorrhizal	Barros et al., 2007
Lactarius salmonicolor	Fungi	Basidiomycota	Agaricomycetes	Russulales	Russulaceae	Lactarius	(L. ex Fr.) S.F.Gray	Mycorrhizal	Heleno et al., 2009
Lepista inversa	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Lepista	(Scop.) Pat	Saprotrophic	Heleno et al., 2009
Lepista nuda	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Lepista	(Bull.) Cooke	Saprotrophic	Barros et al., 2008b
Leucopaxillus giganteus	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Leucopaxillus	(Sowerby) Singer	Saprotrophic	Barros et al., 2007
Lycoperdon molle	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Agaricaceae	Lycoperdon	Pers.	Saprotrophic	Barros et al., 2008b
Marasmius oreades	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Marasmiaceae	Marasmius	(Bolton) Fr	Saprotrophic	Barros et al., 2008a
Ramaria botrytis	Fungi	Basidiomycota	Agaricomycetes	Gomphales	Gomphaceae	Ramaria	(Pers.) Ricken	Mycorrhizal	Barros et al., 2008b
Russula caerulea	Fungi	Basidiomycota	Agaricomycetes	Russulales	Russulaceae	Russula	(Pers) Fr.	Mycorrhizal	Determined herein (Annex 2)
Russula delica	Fungi	Basidiomycota	Agaricomycetes	Russulales	Russulaceae	Russula	Fr.	Mycorrhizal	Heleno et al., 2009
Sarcodon imbricatus	Fungi	Basidiomycota	Agaricomycetes	Thelephorales	Bankeraceae	Sarcodon	(L.) P. Karst.	Mycorrhizal	Barros et al., 2007
Suillus collinitus	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Suillaceae	Suillus	(Fr.) Kuntze	Mycorrhizal	Heleno et al., 2010
Suillus luteus	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Suillaceae	Suillus	(L.: Fries) Gray	Mycorrhizal	Reis et al., 2011a
Suillus mediterraneencis	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Suillaceae	Suillus	(Jacquet & J. Blum) Redeuilh	Mycorrhizal	Heleno et al., 2009
Tricholoma atrosquamosum	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Tricholoma	(Cheval) sacc.	Mycorrhizal	Determined herein (Annex 2)
Tricholoma imbricatus	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Tricholoma	Fr	Mycorrhizal	Heleno et al., 2009
Tricholoma portentosum	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Tricholoma	(Fr.) Quél.	Mycorrhizal	Barros et al., 2007

2. PHENOLIC PROFILE OF THE WILD EDIBLE MUSHROOMS

Protocatechuic, *p*-hydroxybenzoic, *p*-coumaric and cinnamic acids were identified and quantified in the analysed samples (eighteen edible species) by comparing their chromatographic characteristics and absorption spectra with that of the standard compounds (Tables 2, Annexes 1 and 2). The other species were chemically characterized by Barros *et al.* (2007). *Fistulina hepatica* showed the highest concentration of phenolic acids (111.72±7.19 mg/Kg dw), mostly due to the contribution of protocatechuic and *p*-hydroxybenzoic acids. Therefore, this indicats that mushrooms have developed chemical defence mechanisms (against insects and microorganisms) analogous to those that have been observed in plants, such as the production of phenolic compounds. In fact, phenolic compounds have been shown to protect the plant cell wall during UV, salt, or pathogenic stress (Signore *et al.*, 1997; Lattanzio *et al.*, 2006).

Other authors (Ribeiro *et al.*, 2007) reported the presence of caffeic, *p*-coumaric and ellagic acids in *F. hepatica* collected in the same region (Bragança, Portugal) in 2004. However, we did not find any of those phenolic compounds in our sample. Instability in the phenol content over time after mushroom collection is often observed, probably due to enzymatic and oxidative decomposition. This fact, together with the different stress conditions to which the mushrooms were submitted, as well as the different extraction methodologies applied (in addition to possible genetic variability), could explain the differences observed between the present study and other studies, in what concerns the phenolic profile of *F. hepatica* (Ribeiro *et al.*, 2007) and *Hydnum repandum* (Puttaraju *et al.*, 2006).

In addition, in a study from our group, it was possible to detect and quantify cinnamic acid in a Portuguese sample of *H. repandum*, while other authors also quantified tannic, gallic and protocatechuic acids in a sample of this mushroom collected in/ India (Puttaraju *et al.*, 2006).

No phenolic acids were detected in *Laccaria amethystina, Lepista inversa and Russula delica*. Nevertheless, Yaltirak and collaborators (2009) found gallic acid, catechin, caffeic acid and rutin in a sample of *R. delica* from Turkey. These authors used an extraction methodology with ethanol in a soxhlet apparatus at 60ºC. We avoided using heat due to the fact that phenolic compounds are unstable and readily become non-antioxidative under heating conditions and in the presence of antioxidants (Yen and Hung, 2000; Barros *et al.*, 2007a). However, the results obtained by Yaltirak and

collaborators (2009) support the view that heat may increase the concentration in phenolics.

In addition, Choi and collaborators (2006) described that heat treatment of Shiitake increased the overall content in free polyphenolic and flavonoid compounds. The authors explained that heat treatment might produce changes in their extractability due to the disruption of the plant cell wall e thus bound polyphenolic and flavonoid compounds may be released more easily, relative to those from raw materials.

Antioxidants and particularly phenolic compounds may decrease the risks of several chronic diseases such as atherosclerosis, cancer, diabetes, aging and other degenerative diseases in humans (Halliwell, 1996). Overall, *F. hepatica* revealed the highest concentration in phenolic compounds. The phenolic profile of this sample and of a sample of *H. repandum* and *R. delica* has already been described (Yaltirak *et al.*, 2009; Puttaraju, *et al.*, 2006).

However, we pointed out some differences between our results and the results obtained by those authors; Puttaraju and collaborators (2006) found in *H. repandum,* collected in India, tannic acid, gallic acid, protocatechuic acid and cinnamic acid and we only identified cinnamic acid. Yaltirak and collaborators (2009) identified in *R. delica* from Turkey the following phenolic compounds: gallic acid, catechin caffeic acid and rutin. Furthermore, our work describes for the first time the identification of phenolic compounds in fourteen other mushroom species.

The edible mushrooms could be directly used in the human diet as nutritional foods to combat oxidative stress, taking advantage on the possible synergistic and/or additive effects of all the compounds present therein (Liu, 2004). On the other hand, inedible species could represent a source of extractable phenolic compounds to be used as additives in the food industry or as components in pharmaceutical and/or cosmetic formulations, due to their well-known antioxidant properties.

Several phenolic compounds were previously identified and quantified in wild mushrooms from Finland, India, Korea and Portugal (Ferreira *et al.*, 2009) but not in the herein studied species. To the best of our knowledge, this is the first report of the presence of individual phenolic compounds in *A. mellea, C. gambosa, C. odora* and *C. comatus*.

3. ACTIVITY ANTIOXIDANT ACTIVITY OF AQUEOUS, ETHANOLIC AND METHANOLIC EXTRACTS OF WILD EDIBLE MUSHROOMS

The antioxidant activity of *Armillaria mellea*, *Calocybe gambosa, Clitocybe odora, Coprinus comatus, Clitocybe alexandri, Lepista inversa* and *Suillus collinitus* was evaluated using radical species generated in the reaction system, such as DPPH radicals (DPPH scavenging activity assay) and linoleate-free radical (β-carotene bleaching inhibition assay), or by using the reducing effect on Fe^{3+}/ferricyanide complex (reducing power assay).

The water soluble polysaccharidic fractions revealed a higher antioxidant activity than the ethanolic fractions, except for *C. comatus* (Table 1, Annex 1). Moreover, the ethanolic fraction of *A. mellea*, *C. gambosa, C. odora and C. comatus* species showed the highest DPPH radical scavenging activity (EC_{50} value 2.56 mg/ml; extract concentration that causes 50% of antioxidant activity) (Table 2, Annex 1). This observation is in agreement with its higher content in phenolic compounds, compared to the other studied mushrooms. The ethanolic fraction studied here gave better results (reducing power 1.61 at 5 mg/ml; DPPH scavenging activity 79.92% at 5 mg/ml, data not shown) than the ethanolic extracts (reducing power 0.45 at 5 mg/ml; DPPH scavenging activity 84.5% at 5 mg/ml) or the hot water extracts (reducing power 0.25 at 5 mg/ml; DPPH scavenging activity 58.9% at 20 mg/ml) previously obtained by other authors from *C. comatus* collected in Taiwan (Tsai *et al.*, 2007).

The water soluble polysaccharidic fraction of *C. odora* showed the lowest EC_{50} value regarding reducing power (0.94 mg/ml) and β-carotene bleaching inhibition (0.27 mg/ml). The ethanolic fraction of *C. gambosa* showed higher EC_{50} values (i.e., lower antioxidant activity) than a commercial sample previously studied by some elements of our group (7.14 mg/ml, 4.31 mg/ml and 2.77 mg/ml for DPPH scavenging activity, reducing power and *b*-carotene bleaching inhibition, respectively). Nevertheless, that sample, previously studied by some elements involved in this thesis, was a crude methanolic extract obtained at room temperature (Queirós *et al.*, 2009) and was not produced following the fractionated procedure with boiling water and ethanol, such as the one used in the present work.

The ethanolic extracts of *L. inversa* and *C. alexandri* gave higher antioxidant activity (lower EC_{50} values; Table 1, Annex 3) than the methanolic extracts, which is in agreement with the highest content in phenolics found in the first extracts. Nevertheless, it was the

polysaccharidic extracts that revealed the most potent antioxidant activity in the three studied assays (EC_{50} < 2.5 mg/ml).

The methanolic extract of *S. collinitus* revealed the lowest antioxidant activity (highest EC_{50} values) in all the assays. The antioxidant properties of ethanolic and boiled water extracts of *S. collinitus* were similar (without significant statistical differences), except for reducing power which was higher in the ethanolic extract (1.3 mg/ml; Table 2, Annex 5). The observed antioxidant potential might be related to the phenolic acids found (Table 1, Annex 5) and their reported chemoprotective effects against oxidative stress-mediated disorders, mainly their free radical scavenging and metal chelating properties (Soobrattee *et al.*, 2005).

4. TUMOUR CELL GROWTH INHIBITORY ACTIVITY OF THE AQUEOUS, ETHANOLIC AND METHANOLIC EXTRACTS OF WILD EDIBLE MUSHROOMS

The effect of the extracts on the growth of four human tumour cell lines was evaluated, according to the procedure adopted in the NCI's *in vitro* anticancer drug screening, which uses sulforhodamine B (SRB) assay to assess cell growth inhibition. This was carried out for thirty five (35) wild edible mushroom extracts (methanolic, ethanolic and boiling water extracts). Results are expressed as GI_{50} (concentrations of extracts that cause 50% cell growth inhibition). These studies were conducted in four human tumour cells lines, selected as being representative of lung, breast, colon and gastric cancer.

The majority of the extracts did not reveal any growth inhibitory potential in tumour cell lines, up to the highest concentration tested (400 µg/ml, Table 6).

Table 6. Effects of the mushroom extracts on the growth of human tumour cell lines. Results are expressed as the average GI_{50} (in µg/ml) of a minimum of 3 independent experiments (except when GI_{50} > 400µg/ml, in which case a minimum of 2 independent experiments were carried out).

Species	Extracts	NCI-H460 (lung cancer)	MCF-7 (breast cancer)	HCT-15 (colon cancer)	AGS (gastric cancer)
Agaricus arvensis	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Agaricus	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml

Species	Extracts	NCI-H460	MCF-7	HCT-15	AGS
		(lung cancer)	(breast cancer)	(colon cancer)	(gastric cancer)
bisporus	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Armillaria mellea	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Boletus edulis	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Calocybe gambosum	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Cantharellus cibarius (comercial)	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Canthrelus cibarius (Silvestre)	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Clitocybe alexandri	Phenolic (methanolic)	34.8 ± 2.8 µg/ml	34.2 ± 1.4 µg/ml	36.9 ± 3.1 µg/ml	36.1 ± 2.3 µg/ml
	Phenolic (ethanolic)	24.8 ± 2.3µg/ml	17.9 ± 1.3 µg/ml	21.7 ± 2.3 µg/ml	26.0 ± 1.3 µg/ml
	Polysaccharidic (boiling water)	24.5 ± 1.8 µg/ml	46.8 ± 1.6 µg/ml	59.1 ± 0.7 µg/ml	51.7 ± 0.9 µg/ml
Clitocybe odora	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Coprinus comatus	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Craterellus cornucopioides	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Fistulina hepatica	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Flammulina velutipes	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Hygrophorus aurantiaca	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Lacaria amethystina	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Laccaria	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml

Species	Extracts	NCI-H460 (lung cancer)	MCF-7 (breast cancer)	HCT-15 (colon cancer)	AGS (gastric cancer)
laccata	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Lactarius deliciosus	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Lactarius salmonicolor	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Lepista inversa	Phenolic (methanolic)	36.3 ± 5.1 µg/ml	45.2 ± 3.1 µg/ml	39.7 ± 4.6 µg/ml	67.4 ± 5.5 µg/ml
	Phenolic (ethanolic)	118.3 ± 2.5 µg/ml	79.1 ± 11.8 µg/ml	42.3 ± 4.5 µg/ml	58.5 ± 3.3 µg/ml
	Polysaccharidic (boiling water)	155.0 ± 3.5 µg/ml	137.4 ± 1.3 µg/ml	77.4 ± 5.5 µg/ml	99.9 ± 7.8 µg/ml
Lepista nuda	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Leucopaxillus giganteus	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Lycoperdon molle	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Marasmius oreades	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Marasmius oreades	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Ramaria botrytis	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Russula delica	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Sarcodon imbricatus	Phenolic (methanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Suillus collinitus	Phenolic (methanolic)	62.5 ± 6.3 µg/ml	25.2 ± 0.16 µg/ml	103.2 ± 9.9 µg/ml	79.2 ± 15.5 µg/ml
	Phenolic (ethanolic)	253.7 ± 2.3 µg/ml	101.8 ± 8.9 µg/ml	139.4± 34.1 µg/ml	170.7 ± 35 µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
Suillus luteus	Phenolic (methanolic)	30.3± 1.1 µg/ml	32.3 ± 5.7 µg/ml	17.8 ± 1.6 µg/ml	30.33 ± 3.1 µg/ml
	Phenolic (ethanolic)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml
	Polysaccharidic (boiling water)	> 400µg/ml	> 400µg/ml	> 400µg/ml	> 400µg/ml

Species	Extracts	NCI-H460 (lung cancer)	MCF-7 (breast cancer)	HCT-15 (colon cancer)	AGS (gastric cancer)
Tricholoma gambosum	Phenolic (methanolic)	> 400μg/ml	> 400μg/ml	> 400μg/ml	> 400μg/ml
	Phenolic (ethanolic)	> 400μg/ml	> 400μg/ml	> 400μg/ml	> 400μg/ml
	Polysaccharidic (boiling water)	> 400μg/ml	> 400μg/ml	> 400μg/ml	> 400μg/ml
Tricholoma imbricatus	Phenolic (methanolic)	> 400μg/ml	> 400μg/ml	> 400μg/ml	> 400μg/ml
	Phenolic (ethanolic)	> 400μg/ml	> 400μg/ml	> 400μg/ml	> 400μg/ml
	Polysaccharidic (boiling water)	> 400μg/ml	> 400μg/ml	> 400μg/ml	> 400μg/ml
Tricholoma portentosum	Phenolic (methanolic)	> 400μg/ml	> 400μg/ml	> 400μg/ml	> 400μg/ml
	Phenolic (ethanolic)	> 400μg/ml	> 400μg/ml	> 400μg/ml	> 400μg/ml
	Polysaccharidic (boiling water)	> 400μg/ml	> 400μg/ml	> 400μg/ml	> 400μg/ml

Note: This is the only table presented in the results and discussion section, since it contains unpublished data. All the other obtained results are presented in the Annexes.

The mushroom species that revealed cell growth inhibitory activity against at least some of the studied human tumour cell lines were: *Clitocybe alexandri, Lepista inversa*, *Suillus collinitus* and *Suillus luteus*.

Indeed, the phenolic (methanolic and ethanolic) and the polysaccharidic (boiling water) extracts from *C. alexandri* and *L. inversa* revealed capacity to inhibit 50% of the growth of all the human tumour cell lines studied, when used in concentrations lower than 160 μg/ml. *C. alexandri* was more potent than *L. inversa* in inhibiting the growth of the four studied human tumour cell lines. For *C. alexandri*, the lowest GI_{50} values were generally obtained with the ethanolic extracts, while for *L. inversa*, methanolic extracts proved to be generally more potent, with the exception of the AGS cells (in which the more potent extract was the ethanolic). For both mushrooms, the polysaccharidic extract was usually the less potent one (Table 2, Annex 3).

Regarding the *S. collinitus*, the methanolic extract was the most potent of the tested extracts in all the tested cell lines, presenting GI_{50} values ranging from 25.2 to 103.2 μg/ml in MCF-7 and HCT-15 cells, respectively. The boiled water extract did not show any effect on the tested cell lines at the studied concentrations (up to 400 μg/ml). The MCF-7 cell line was the most susceptible (i.e. presented the lowest GI_{50}) to the *S. collinitus* methanolic extract (Table 3, Annex 5).

Regarding *S. Luteus*, the methanolic extract was the only one that inhibited cell growth in all the cell lines tested and up to the maximum concentration tested (up to 400 μg/ml). The HCT-15 cell line was the most susceptible (presented the lowest GI_{50}) to this extract (Table 6).

5. *THE CLITOCYBE ALEXANDRI* ETHANOLIC EXTRACT AND THE *SUILLUS COLLINITUS* METHANOLIC EXTRACT IN CELL CYCLE AND APOPTOSIS IN LUNG AND BREAST CANCER CELL LINES, RESPECTIVELY

Two of the most active samples, *Clitocybe alexandri* ethanolic extract and *Suillus collinitus* methanolic extract, were chosen to be further investigated regarding some elucidation of their possible mechanism of action.

Regarding the *C. alexandri* ethanolic extract

The NCI-H460 cell line was incubated with the GI_{50} (24.8 μg/ml) or $2\times GI_{50}$ (49.6 μg/ml) concentrations of the *C. alexandri* ethanolic extract, for 48 h, and the effects of this extract on the normal cell cycle distribution and induction of apoptosis were studied.

The analysis of the effect of the ethanolic extract of *C. alexandri* on the cell cycle profile was performed by flow cytometry. Results show a dose-dependent increase in the percentage of cells in the S-phase of the cell cycle, with a concomitant decrease in the percentage of cells in the G1 and G2/M phases (Figure 1, Annex 4). Therefore, the *C. alexandri* extract seems to be an inducer, in these non-small cell lung cancer cells, of an increase in the percentage of cell in the S-phase of the cell cycle following 48 h of treatment.

Additionally, it was investigated whether *C. alexandri* induced apoptosis in the NCI-H460 cell line, by the annexin V-FICT/PI flow cytometry assay. NCI-H460 cells treated with twice the GI_{50} concentration of the ethanolic extract of *C. alexandri* for 48 h presented a significant increase in the percentage of apoptotic cells (28.6% ± 0.9%), when compared to the blank (untreated) cells (6.6% ± 0.3%).Nonetheless, cells treated with the GI_{50} concentration of the extract for 48 h only presented 7.3% ± 2.1% of apoptotic cells (similar to the blank treatment i.e. similar to untreated cells).

Furthermore, the effect of this *C. alexandri* mushroom extract on the expression of some proteins involved in the apoptotic process was determined by Western blot. Results showed that treatment of NCI-H460 cells with the GI_{50} concentration of the extract for 48h caused an increase in the levels of wt p53, cleaved caspase-3 and cleaved PARP (Figure 2, Annex 4), consistent with apoptotic cell death.

Regarding the *S. collinitus* methanolic extract

The MCF-7 cell line was incubated with the GI_{50} (25.2 μg/ml) or twice the GI_{50} (50.4 μg/ml) concentrations of the *S. collinitus* methanolic extract, for 48 h, and the effect of this treatment on the normal cell cycle distribution and induction of apoptosis were studied, by flow cytometry.

The results show that both the GI_{50} concentration and $2\times GI_{50}$ concentration of this extract increased the number of cells in the G1 phase of the cell cycle and concomitantly decreased the percentage of cells in the S phase of the cell cycle (Figure 2, Annex 5).

Additionally, it was investigated whether *S. collinitus* methanolic extract induced apoptosis in the MCF-7 cell line, using the annexin V-FICT/PI flow cytometry assay. MCF-7 cells treated with the methanolic extract of *S. collinitus* for 48 h presented an increase in the percentage of apoptotic cells, from 6.0±0.2% in untreated (blank) cells, to 15.3±2.0% in cells treated with the GI_{50} concentration and to 16.3±2,0 % in cells treated with $2\times GI_{50}$ concentration.

Furthermore, the effect of this mushroom extract on the expression of some proteins involved in the cell cycle and/or apoptotic process was determined, by Western blot. Results show that treatment of MCF-7 cells with the GI_{50} concentration of the extract caused a strong increase in the levels of p53. This effect was even stronger when cells were treated with $2\times GI_{50}$ concentration, suggesting that the effect is concentration-dependent. Accordingly, the levels of p21, whose expression is regulated by p53 and related to cell cycle progression, were also increased in a concentration dependent manner (with the GI_{50} and with the $2\times$ GI_{50} concentrations). Finally, treatment of these cells with the extract caused a decrease in the levels of XIAP and Bcl-2 and a concentration-dependent increase in the levels of cleaved PARP, which is consistent with an apoptotic process of cell death (Figure 4, Annex 5).

The effect of treating MCF-7 cells concomitantly with *S. collinitus* (methanolic extract) and etoposide was also studied, by verifying the % of cell growth upon a 48 h incubation with the previously determined approximate GI_{50} concentration of etoposide (1 μM) and with the GI_{50} (25.2 μg/ml) of the extract. The combined use of the methanolic extract and etoposide caused a greater reduction in the % of cell growth, when compared to either of them used individually, indicating the potential benefit of this combination (Figure 5, Annex 5).

6. CYTOTOXIC POTENTIAL IN TUMOUR CELL LINES OF SOME PHENOLIC ACIDS IDENTIFIED IN THE MOST ACTIVE WILD EDIBLE MUSHROOMS

The *in vitro* cell growth inhibitory activity of the compounds (protocatechuic, *p*-hydroxybenzoic, and cinnamic acids) identified in the two most active wild mushrooms (*C. alexandri* and *S. collinitus*) was evaluated with the SRB assay, after a continuous treatment of NCI-H460 cells during 48 h with these compounds.

Cinnamic acid was found to be the most potent compound regarding cell growth inhibition even though the GI_{50} was quite high (GI_{50} value 845.9 ± 97.5 μM) (Figure 4, Annex 4).

Protocatechuic acid revealed a GI_{50} value of 1616.9 ± 75.3 μM, while *p*-hydroxybenzoic acid did not show any activity up to the highest tested concentration (3000 μM) (Figure 4, Annex 4).

The effect of the individual and combined treatment with these identified compounds was also tested, by verifying the number of viable cells upon a 48 h incubation with the GI_{50} concentrations of cinnamic and protochatequic acids and 3000 μM of *p*-hydroxibenzoic acid (the maximum concentration tested in the SRB, since the GI_{50} was not obtained even with this high concentration). Results were compared with those obtained with DMSO control and blank treatment (i.e. cells incubated with complete medium). Treatment with the GI_{50} concentration of cinnamic and protochatequic acids caused a reduction in the number of viable cells up to ≈ 50 %, as expected to occur with the GI_{50} concentration of any compound (Figure 5, Annex 4).In addition, *p*-hydroxibenzoic acid did not cause any a significant reduction in the viable cell number, as expected from the results previously obtained with the SRB assay.

In addition, it was verified that the concomitant use of the three compounds provided the strongest decrease in the viable number of NCI-H460 cells, suggesting a potential concomitant effect of those compounds (Figure 5, Annex 4).

CHAPTER IV.

CONCLUSIONS

Among the 35 wild edible mushrooms collected in the Northeast of Portugal and properly identified, 114 extracts (phenolic and polysaccharidic) were prepared for analysis of antioxidant activity and/or cell growth inhibitory activity in tumour cell lines. None of these species had ever been studied with regard to the latter activity. Three species revealed growth inhibitory activity in the studied human tumour cell lines (NCI-H460 - lung cancer, MCF-7 -breast cancer, HCT-15 -colon cancer and AGS - gastric cancer): *C. alexandri* ethanolic extract, *L. inversa* methanolic extract and *S. collinitus* methanolic extract.

The most susceptible cell lines were NCI-H460 for *C. alexandri* (GI_{50} 26.0 ± 1.3 μg/ml) and MCF-7 *S. collinitus* (GI_{50} 25.2 ± 0.16 μg/ml) extracts.

C. alexandri ethanolic extract induced an S-phase cell cycle arrest and increased the percentage of apoptotic cells, in the NCI-H460 cell line.

S. collinitus methanolic extract induced a slight increase in the number of cells in G1, with a concomitant decrease in the percentage of cells in the S phase of the cell cycle and an increase in the percentage of apoptotic cells, in the MCF-7 cell line.

The combined use of the *S. collinitus* methanolic extract and etoposide (a known cytotoxic drug used in the treatment of some cancers) caused a greater decrease in the percentage of cell growth, when compared to either of them used individually, indicating the potential benefit of this combination.

The analysed mushroom species also provided interesting antioxidant potential, mainly the polysaccharidic extract of *L. inversa* which showed the highest DPPH radical scavenging activity, reducing power and β-carotene bleaching inhibition.

The tested extracts were chemically characterized and protocatechuic, *p*-hydroxybenzoic, *p*-coumaric and cinnamic acids were the main compounds identified on the phenolic (methanolic and ethanolic) extracts, while mannitol, trehalose and arabinose were the main sugars found in the polysaccharidic (boiling water) extracts after hydrolysis.

The individual compounds identified in the extracts were submitted to a screening of tumour cell growth inhibitory activity, but only the phenolic acids and a related compound, cinnamic acid, presented some (although modest) activity. This compound was found to be the most potent studied one, regarding cell growth inhibition in the NCI-H460 cell line.

The effect of the individual and combined treatment with the identified phenolic compounds was also evaluated. Treatment with the GI_{50} concentration of cinnamic and protochatequic acids caused a statistically significantly reduction in the number of viable cells (up to ≈50%), as expected to occur with the GI_{50} concentration of any compound. In addition, *p*-hydroxybenzoic acid did not show any significantly reduction in the viable cell number, as expected from the results previously obtained with SRB assay. Nevertheless, it was verified that the concomitant use of the three compounds provided the strongest decrease in the viable cell number, suggesting a possible concomitant effect of those compounds.

In conclusion, the results obtained in this thesis help to clarify the bioactive potential of wild edible mushrooms from the Northeast of Portugal. This study allowed to identify some species with antioxidant or tumour cell growth inhibitory potential. Nonetheless, more studies are needed to be carried out in order to elucidate the mechanism of action of the best extracts and the contribution of each of the identified molecules towards the observed phenotype. In addition, the isolation of more compounds from these extracts and their further study regarding these activities needs to be carried out.

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ANNEXES

ANNEX 1 - CHEMICAL COMPOSITION OF WILD EDIBLE MUSHROOMS AND ANTIOXIDANT PROPERTIES OF THEIR WATER SOLUBLE POLYSACCHARIDIC AND ETHANOLIC FRACTIONS.

ANNEX 2 - PHENOLIC PROFILE OF SEVENTEEN PORTUGUESE WILD MUSHROOMS.

ANNEX 3 - WILD MUSHROOMS *CLITOCYBE ALEXANDRI* AND *LEPISTA INVERSA*: *IN VITRO* ANTIOXIDANT ACTIVITY AND GROWTH INHIBITION OF HUMAN TUMOUR CELL LINES.

ANNEX 4 - *CLITOCYBE ALEXANDRI* EXTRACT INDUCES CELL CYCLE ARREST AND APOPTOSIS IN A LUNG CANCER CELL LINE: IDENTIFICATION OF PHENOLIC ACIDS WITH CYTOTOXIC POTENTIAL.

ANNEX 5 - *SUILLUS COLLINITUS* METHANOLIC EXTRACT INCREASES P53 EXPRESSION AND CAUSES CELL CYCLE ARREST AND APOPTOSIS IN A BREAST CANCER CELL LINE