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Departamento de Química Analítica, Nutrición y Bromatología

CARACTERIZACIÓN QUÍMICA Y PROPIEDADES BIOACTIVAS DE HONGOS SILVESTRES PORTUGUESES COMESTIBLES

CHEMICAL CHARACTERIZATION AND BIOACTIVE PROPERTIES OF PORTUGUESE WILD EDIBLE MUSHROOMS

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ABSTRACT

Nineteen different mushroom species (*Agaricus arvensis*, *Agaricus bisporus*, *Agaricus romagnesii*, *Agaricus silvaticus*, *Agaricus silvicola*, *Cantharellus cibarius*, *Hypholoma fasciculare*, *Lactarius deliciosus*, *Lactarius piperatus*, *Lepista nuda*, *Leucopaxillus giganteus*, *Lycoperdon molle*, *Lycoperdon perlatum*, *Macrolepiota mastoidea*, *Macrolepiota procera*, *Ramaria botrytis*, *Sarcodon imbricatus*, *Tricholoma acerbum* and *Tricholoma portentosum*) from Northeast of Portugal, one of the European regions with higher wild edible mushrooms diversity, were evaluated for their chemical composition, nutritional value and bioactive properties (antioxidant and antimicrobial activities), in order to valorise mushrooms as a source of nutrients and nutraceuticals.

The analysis of nutrients included determination of proteins, fats, ash, and carbohydrates, particularly sugars by HPLC-RI. The analysis of nutraceuticals included determination of fatty acids by GC-FID, and other phytochemicals such as tocopherols, by HPLC-fluorescence, phenolic compounds by HPLC-DAD-ESI/MS, carotenoids and ascorbic acid, by spectrophotometric techniques. The antioxidant activity was screened through chemical and biochemical assays. The chemical assays allowed an evaluation of their reducing power, radical scavenging activity and inhibition of β-carotene bleaching, while biochemical assays evaluated the lipid peroxidation inhibition capacity, using erythrocytes and brain cells as models. The antimicrobial activity was evaluated using clinical isolates or collection microorganisms (Gram positive and Gram negative bacteria and fungi).

The macronutrient profile in general revealed that the wild mushrooms were rich sources of protein and carbohydrates and had low amounts of fat. The analysis of fatty acid composition allowed the quantification of twenty three fatty acids. Unsaturated fatty acids and, in particular, oleic and linoleic acids, were predominant. Mannitol and trehalose were the most abundant sugars. The analysed mushrooms also contain very useful phytochemicals such as phenolic compounds, tocopherols, ascorbic acid, and carotenoids. Particularly, four phenolic acids (protocatechuic, *p*-hydroxybenzoic acid, *p*-coumaric and cinnamic acid) and two vanillic acid isomers were detected, identified and quantified, as also three of the tocopherols vitamers (α-, β-, γ- tocopherol); no tocotrienols were detected. All the species proved to have antioxidant properties,

namely radical scavenging activity and lipid peroxidation inhibition capacity. *Ramaria botrytis* was the most efficient species presenting the lowest EC_{50} values in the chemical and biochemical assays, which can be related to their higher content in bioactive compounds. The majority of the species revealed antimicrobial activity selectively against Gram + bacteria, in some cases, with lower minimum inhibitory concentration than the standards.

Processing and cooking practices had a determining influence on chemical composition and antioxidant properties. Cooked samples showed lower nutrients concentrations and lower antioxidant activity than either dried or frozen samples. Nevertheless, for fatty acids and sugar individual profiles, only cooking procedures seemed to be relevant, the cooked samples presenting higher MUFA, and lower PUFA and sugars contents. The fruiting body maturity stage proved to have influence on chemical composition and bioactivity of the wild mushrooms; mature carpophorus with mature spores is not recommended for nutritional and medicinal proposals.

In addition to dried mushrooms, alternative or substitute mushroom products are mycelia that could also be used as food and food-flavouring material, or in the formulation of nutraceuticals and functional foods. In order to explore it, mycelium of *Leucopaxillus giganteus* was produced using different pH, carbon and nitrogen sources in the culture medium, and evaluated for their bioactive properties. The antioxidants concentration increased along the growth time as a response to the oxidative stress and subsequent free radicals production. The aldohexose glucose and diammonium phosphate proved to be the most appropriate carbon and nitrogen sources to increase antioxidant activity, leading to the highest phenols content and lowest EC_{50} values.

Public health authorities consider prevention and treatment with nutraceuticals/phytochemicals a powerful instrument in maintaining and promoting health, longevity and life quality. The beneficial effects of nutraceuticals will undoubtedly have an impact on nutritional therapy; they also represent a growing segment of today's food industry. Mushrooms might be used directly in diet and promote health, taking advantage of the additive and synergistic effects of all the bioactive compounds present. Therefore, the ongoing research will lead to a new generation of foods, and will certainly promote their nutritional and medicinal use.

RESUMEN

Diecinueve especies diferentes de setas silvestres (*Agaricus arvensis*, *Agaricus bisporus*, *Agaricus romagnesii*, *Agaricus silvaticus*, *Agaricus silvicola*, *Cantharellus cibarius*, *Hypholoma fasciculare*, *Lactarius deliciosus*, *Lactarius piperatus*, *Lepista nuda*, *Leucopaxillus giganteus*, *Lycoperdon molle*, *Lycoperdon perlatum*, *Macrolepiota mastoidea*, *Macrolepiota procera*, *Ramaria botrytis*, *Sarcodon imbricatus*, *Tricholoma acerbum* and *Tricholoma portentosum*) del Nordeste de Portugal, una de las regiones europeas con mayor diversidad hongos silvestres comestibles, fueron evaluados con relación a su composición química, valor nutritivo y propiedades bioactivas (actividades antioxidante y antimicrobiana), con vistas a su valorización como fuente de nutrientes y nutracéuticos.

El análisis de nutrientes incluyó la determinación de proteínas, grasas, cenizas, hidratos de carbono, y particularmente azúcares por HPLC-RI. El análisis de nutracéuticos incluyó la determinación de ácidos grasos por GC-FID y otros fitoquímicos, como tocoferoles por HPLC-fluorescencia, compuestos fenólicos por HPLC-DAD-ESI/MS, carotenoides y ácido ascórbico por espectrofotometría. La actividad antioxidante se valoró mediante ensayos químicos y bioquímicos. Los ensayos químicos consistieron en la evaluación del poder reductor, inhibición del radical libre DPPH e inhibición de la decoloración del β-caroteno, mientras que los ensayos bioquímicos evaluaron la capacidad de inhibición de la peroxidación lipídica, utilizando como modelos eritrocitos y células del cerebro. La actividad antimicrobiana fue evaluada utilizando aislados clínicos y microorganismos de colección (bacterias Gram positivas y Gram negativas y hongos).

El perfil de macronutrientes reveló que, en general, los hongos silvestres son fuentes ricas de proteínas e hidratos de carbono y que poseen poca cantidad de grasa. El análisis de la composición de ácidos grasos permitió la cuantificación de veintitrés ácidos grasos. Los ácidos grasos insaturados y, en particular, los ácidos oleico y linoleico, eran predominantes. Manitol y trehalosa fueron los azúcares más abundantes. Las setas analizadas también contienen fitoquímicos tales como compuestos fenólicos, tocoferoles, ácido ascórbico y carotenoides. En particular, fueron detectados, identificados y cuantificados cuatro ácidos fenólicos (ácidos protocatéquico, *p*-hidroxibenzoico, *p*-cumárico y cinámico) y dos isómeros del ácido vanílico y también

tres isómeros de tocoferoles (α, β, de γ-tocoferol), mientras que no se detectaron tocotrienoles. Todas las especies mostraron propiedades antioxidantes, especialmente capacidad para inhibir radicales libres y la peroxidación lipídica. *Ramaria botrytis* fue la especie más eficiente en este sentido, presentando los valores más bajos de EC_{50} en los ensayos químicos y bioquímicos, lo que puede estar relacionado con su mayor contenido en compuestos bioactivos. La mayoría de las especies mostraron actividad antimicrobiana selectiva contra las bacterias Gram positivas, en algunos casos, con concentraciones mínimas inhibitorias menores que las de los compuestos usados como referencia.

El procesado y las prácticas culinarias ejercen una influencia determinante en la composición química y propiedades antioxidantes de las setas estudiadas. Las muestras cocinadas tenían menor concentración de nutrientes y menor actividad antioxidante que las muestras secas o congeladas. Sin embargo, los perfiles individuales de ácidos grasos y azúcares sólo se vieron afectados en las muestras cocinadas, las cuales presentaban niveles mayores de ácidos grasos monoinsaturados e inferiores de poliinsaturados y de azúcares.

La maduración del cuerpo fructificante también demostró tener influencia en la composición química y en la bioactividad de las setas silvestres; así, los carpóforos maduros con esporas maduras no pueden ser recomendados nutricional ni medicinalmente.

Un producto alternativo a las setas desecadas son los micelios, que también podrían ser utilizados como alimento o ingrediente, o en la formulación de nutracéuticos y alimentos funcionales. Para explorar esta posibilidad, se obtuvieron micelios de *Leucopaxillus giganteus* utilizando diferentes pH, fuentes de carbono y nitrógeno en el medio de cultivo, y posteriormente se evaluaron sus propiedades bioactivas. La concentración de antioxidantes aumentó a lo largo del tiempo decrecimiento como respuesta al estrés oxidativo y, por tanto, la producción de radicales libres. La aldohexosa glucosa y el fosfato diamónico demostraron ser respectivamente las fuentes de carbono y de nitrógeno más apropiadas para aumentar la actividad antioxidante, dando lugar al más alto contenido de compuestos fenólicos y valores más bajos de EC_{50}.

El empleo de nutracéuticos/fitoquímicos puede ser un instrumento eficaz en el mantenimiento y la promoción de la salud, longevidad y calidad de vida. Los efectos beneficiosos de nutracéuticos, sin duda, no sólo tienen un impacto en la terapia nutricional, sino que también representan un segmento creciente de la actual industria

alimentaria. En este sector podrían ubicarse las setas, cuyo empleo como parte de la dieta con fines de promoción de la salud podría ser tenido en cuenta a la vista de la variedad de compuestos bioactivos que contienen y los posibles efectos aditivos o sinérgicos que pueden existir entre ellos. La investigación en curso trata de explorar esta alternativa para, en definitiva, promover nuevas generaciones de alimentos con un valor añadido basado en sus propiedades nutritivas y medicinales.

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ABBREVIATIONS

Standard chemical symbols and SI units are used without definition.

AAPH 2,2' azobis (2-amidopropane) dihydrochloride
AH Primary antioxidants
ANOVA Analysis of variance
AOC Antioxidant capacity
AR Analytical reagent
ArOH Phenolic antioxidant
ATP Adenosine triphosphate
BHA Butylated hydroxyanisole
BHT Butylated hydroxytoluene
ºC Degrees Celsius
CAT Catalase
CFU/mL Colony forming unit/millilitre
DAD Diode array detector
DNA Deoxyribonucleic acid
$DPPH^{\bullet}$ 2,2-diphenyl-1-picrylhydrazyl radical
ESI/MS Electrospray ionization tandem mass spectrometry
ET Electron transfer
EC_{50} Effective concentration providing 50% of antioxidant activity
FC Folin-Ciocalteu (reagent)
FAD Flavin adenine dinucleotide
Fe^{2+} Ferrous ion
Fe^{3+} Ferric ion
FID Flame ionization detector
g Gram
GC Gas chromatography
GAEs Gallic acid equivalents
GSH Glutathione
GPH-R Glutathione redutase
GSH-Px Glutathione peroxidases
GST Glutathione-S-transferases
HAT Hydrogen atom transfer
H_2O_2 Hydrogen peroxide
$HO^{\bullet}$ Hydroxyl radical
$HOO^{\bullet}$ Perhydroxyl radical
$HO_2^{\bullet}$ Hydroperoxyl radical
HPLC High performance liquid chromatography
Kg Kilogram
KNO_3 Potassium nitrate
hr Hour
L Litre
$L^{\bullet}$ Lipid radical
LDL Low-density lipoprotein
LH Polyunsaturated lipid
LOOH Lipid hydroperoxides
$LOO^{\bullet}$ Lipid peroxyl radical

LPO Lipid peroxidation
LPS Lipopolysaccharides
MDA Malondialdehyde
MIC Minimum inhibitory concentration
min Minute/s
mg Milligram
mL Millilitre
MUFA Monounsaturated fatty acids
MMN Melin-Norkans agar
NADPH Nicotinamide adenine dinucleotide phosphate-oxidase
$NaNO_2$ Sodium nitrite
nd not done
NH_4NO_3 Ammonium nitate
$(NH_4)_2HPO_4$ Ammonium hydrogenophosphate
$NO^{\bullet}$ Nitric oxide
O_2 Oxygen
$O_2^{\bullet-}$ Superoxide anion radical
$OH^{\bullet}$ Hydroxyl radical
$ONOO^-$ Peroxynitrite anion
PDA Potato dextrose agar
PG Propyl gallate
PUFA Polyunsaturated fatty acids
ROS Reactive oxygen species
$ROO^{\bullet}$ Peroxyl radicals
RI Refraction index detector
rpm Revolutions per minute
Rt Retention time
RNS Reactive nitrogen species
RSA Radical scavenging activity
s Second/s
SAS Statistical Analysis Software
SOD Superoxide dismutase
SFA Saturated fatty acids
TAA Total antioxidant activity
TBA Thiobarbituric acid
TBARS Thiobarbituric acid reactive substance
TBHQ *tert*-Butylhydroquinone
UV Ultra-Violet
UFA Unsaturated fatty acids
VIS Visible

CHAPTER I

INTRODUCTION

1.1. Medicinal properties of natural products

For thousands of years medicine and natural products have been closely linked through the use of traditional medicines and natural poisons. Natural products represent a rich source of biologically active compounds and are an example of molecular diversity, with recognized potential in drug discovery and development (Cragg et al., 1997; Grabley and Thiericke, 1998; Grabley and Thiericke, 1999; Harvey, 2000; Newman et al., 2000). Numerous reviews have described the importance of compounds derived from microorganism, plant and animal sources to treat human diseases (Newman et al., 2003; Butler, 2004; Butler, 2005). In the areas of cancer and infectious disease, 60% and 75% of new drugs, respectively, originated from natural sources between 1981 and 2002 (Butler, 2004). Between 2001 and 2005, 23 new drugs derived from natural products were introduced for the treatment of disorders such as bacterial and fungal infections, cancer, diabetes, dyslipidemia, atopic dermatitis, Alzheimer's disease and genetic diseases such as tyrosinaemia and Gaucher disease (Butler, 2005). Over half of the world's top 25 best-selling pharmaceutical drugs in 1991 owed their origin to natural products (Kinghorn and Balandrin, 1993; Kinghorn, 2002); these drugs include the ACE (Angiotensin-Converting Enzyme) inhibitors enalapril and captopril, the non-steroidal anti-inflammatory agents diclofenac and naproxen, the antibiotics amoxicillin/clavulanic acid, the β2-agonist salbutamol and the immunosuppressant ciclosporin. Between 1960 and 1986 more than 35,000 species and 108,330 extracts were screened against murine tumours and 11 compounds were approved for extensive tumour panel testing, to come into clinical use (Phillipson, 2007).

The World Health Organization estimated that 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts and their active components (Winston, 1999). Clinical, pharmacological and chemical studies of traditional drugs derived from higher plants were the basis of most early medicines, representing around 25% of the total number of clinically used drugs such as aspirin, atropine, codeine, digitoxin, digoxin, morphine, quinine and pilocarpine (Badalyan, 2003; Phillipson, 2007). Plants have been a major focus of investigations for novel biologically active compounds from natural resources and in recent years pharmaceutical companies have spent a lot of time developing these natural products to produce more affordable and cost effective remedies (Lam, 2007;

Mishra et al., 2007; Phillipson, 2007). However, some plant species have become threatened due to overcollection from the wild and the sustainable usage of natural resources is currently questioned by ecologists (Cragg et al., 1997; Phillipson, 2007).

Filamentous fungi are the producers of some of the most powerful secondary metabolites, which have been researched and developed into therapeutic agents. Two major classes of compounds from filamentous fungi that have been developed into drugs are the β-lactam antibiotics and statin drugs. β-lactam antibiotics have stemmed from the discovery of the first antimicrobial agent from a species of *Penicillium* by Sir Alexander Fleming in the 1920s (Fleming, 1929). This led to the research and development of the antibiotic (penicillin) by Florey and his team (Florey et al., 1949) and opened the way for the development of other antibiotics and therapeutic compounds. Penicillin still remains among the most active and least toxic antibiotics. Statins, another example of natural products successfully developed as drugs, are currently used in reducing the risks for hypercholesterolaemia and coronary heart disease (Shu, 1998). The first statin, mevastatin (compactin), was discovered from a species of fungus *Penicillium* (Endo et al., 1976). Since then, other statin inhibitors have been isolated from different sources, such as lovastatin from *Aspergillus terrus* (Alberts et al., 1980), or obtained by the conversion of statins into more active analogues, such as pravastatin (Haruyama et al., 1986) and simvastatin (Hoffman et al., 1986).

1.2. Fungi Kingdom

The organisms of the fungal lineage include mushrooms, rusts, smuts, puffballs, truffles, morels, molds, and yeasts, as well as many less well-known organisms (Alexopoulos et al., 1996). Fungi were classified into their own separate kingdom from plants by Whittaker in 1969. This was established on the basis that not one fungus is photosynthetic, fungi must absorb nutrients produced by other organisms and they differ from plants in their cell wall composition, in their body structure and in their modes of reproduction (Whittaker, 1969).

Fungi are characterized by non-motile bodies (thalli) constructed of apical elongating walled filaments (hyphae), a life cycle with sexual and asexual reproduction, usually from a common thallus, haploid thalli resulting from zygotic meiosis, and heterotrophic nutrition. Spindle pole bodies, not centrioles, usually are associated with the nuclear

envelope during cell division. The characteristic wall components are chitin (beta-1,4-linked homopolymers of N-acetylglucosamine in microcrystalline state) and glucans primarily alpha-glucans (alpha-1,3- and alpha-1,6- linkages) (Griffin, 1994; Alexopoulos et al., 1996). The Fungi kingdom was further classified into four different phyla: 1) Basidiomycota (club fungi), 2) Ascomycota (sac fungi), 3) Zygomycota (conjugation fungi), and 4) Deuteromycota (imperfect fungi).

Species in the Basidiomycota phylum include species from the Basidiomycetes class that have macroscopic fruiting bodies, large enough to be seen with the naked eye. Many mushrooms in this phylum look like umbrellas growing from the ground. Among the more famous genera in this phylum are *Agaricus* (including the supermarket variety of button mushrooms), *Amanita* (including species that are deadly, delicious, or even hallucinogenic), *Boletus* (best known for the King Bolete), and *Cantherellus* (known for the delicious and beautiful Chanterelle). The prized Morel and Truffle mushrooms are species of the Ascomycota phylum; *Saccharomyces cerevisiae* is another specie from this phylum which is valued for its biotechnological applications. The best known specie of Zygomycota phylum, of around 600 species, is black bread mold, such as *Rhizopus stolonifer*. Deuteromycota, the imperfect fungi, include genera such as *Trichophyton* (Athlete's foot), *Penicillium* (Penicillin), and *Candida albicans* ("Yeast" infections) (Arora, 1986; Margulis and Schwartz, 1988; Alexopoulos et al., 1996).

Since the discovery of Penicillin from the fungus *Penicillium* (Fleming, 1929), which belongs to the imperfect fungi, there has been much focus on the production of antibacterial agents from the filamentous fungi within this division (Al-Hilli and Smith, 1992; Larena and Melgarejo, 1996; Florianowicz, 1998; Fischer et al., 2000; Mayordomo et al., 2000; Rodrigues et al., 2000). Although there is early reference to the antibacterial activities exhibited by fungi that belong to the division Basidiomycota (Robbins et al., 1947; Brian, 1951; Takeuchi, 1969), it is within the last twenty years that a broader range of genera, species and isolates from within this division has been explored in more detail for antibiotic properties (Anke et al., 1980; Colleto and Mondino, 1991; Lorenzen and Anke, 1998; Wasser and Weis, 1999a; Rosecke and Konig, 2000; Wasser, 2002).

1.3. Pharmacological potential of Basidiomycetes

When considering natural substances for investigation of medicinal properties, Baker et al. (1995) suggested that one should consider the evidence regarding the traditional usage of the substance by indigenous populations, the abundance of the species in nature and the sustainable utilisation of the species. These criteria were suggested for the discovery of natural products from the Plant Kingdom. However, fungi, a separate Kingdom from plants, should also fulfil these criteria and most importantly, the sustainability of fungi can be achieved by using artificial cultivation techniques. Another extremely important criterion when searching for novel bioactive compounds is the uniqueness of the organism and its potential to produce secondary metabolites (Donadio et al., 2002a).

In fact, in addition to the ancient utilization of plants the medicinal use of mushrooms has also a very long tradition. Among the large resources of fungi, higher Basidiomycetes, especially mushrooms, are unlimited sources of therapeutically useful biologically active agents (Mizuno, 1995; Wasser, 1995; Wasser, 2002). In particular, mushrooms useful against cancers of the stomach, esophagus, lungs, etc. are known in China, Japan, Korea, Russia, United States and Canada (Wasser and Weis, 1999a).

"Mushroom" is not a taxonomic category. The term "mushroom" is used here according to the definition of Chang and Miles as "a macrofungus with a distinctive fruiting body, which can be either hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand" (Chang and Miles, 1992). From a taxonomic point of view, mainly basidiomycetes but also some species of ascomycetes belong to mushrooms. The number of mushroom species on the earth is estimated to be 140,000, suggesting that only 10% are known. Assuming that the proportion of useful mushrooms among the undiscovered and unexamined mushrooms will be only 5%, this implies 7,000 yet undiscovered species of possible benefit to mankind (Hawksworth, 2001).

The higher Basidiomycetes include about 10,000 species from 550 genera and 80 families in the Basidiomycetes class with macroscopic fruiting bodies; approximately 700 species of higher Basidiomycetes have been found to possess significant pharmacological activities (Mizuno, 1995; Wasser, 1995; Wasser and Weis, 1999a). The macrofungi can be divided into four groups: (1) edible flesh, e.g. *Agaricus*; (2)

medicinal, e.g. *Ganoderma*; (3) poisonous, e.g. *Amanita*; and (4) miscellaneous, where the properties are less well defined (Liu et al., 1995).

It is not surprising that mushrooms are a source of many biologically active compounds. Mushrooms manage to grow in darkness and dampness in highly competitive environments and protect themselves from hordes of attacking microbes by developing natural protective substances. Modern scientific studies on medicinal mushrooms have expanded exponentially during the last two decades and scientific explanation to show mushrooms derived compounds function in human system are increasingly being established (Zaidman et al., 2005). Fungal fruiting bodies, fungal mycelium or the culture fluid in which the mycelium has been cultivated may all be explored for biological activity. More recently, some species of edible higher Basidiomycetes have been found to markedly inhibit the growth of different kinds of tumors. There are approximately two hundred species of higher Basidiomycetes that have found to possess this activity (Ponchet et al., 1982). Both cellular components and secondary metabolites of a large number of mushrooms have been shown to affect the immune system of the host and therefore might be used to treat a variety of diseases (Wasser and Weis, 1999a). Those which appear to enhance or potentiate host resistance are being sought for the treatment of cancer, immunodeficiency diseases (including AIDS) or generalized immunosuppression after drug treatment (Pujol et al., 1990; Clericuzio et al., 2004).

Medicinal mushrooms have an established history of use in traditional oriental medicine, where most medicinal mushroom preparations are regarded as a tonic, that is, they have beneficial health effects without negative side-effects and can be used on a regular, ongoing basis without harm. Often blends of various medicinal mushrooms are used for maximum benefit. Many traditionally used mushrooms from genera, *Auricularia*, *Flammulina*, *Ganoderma*, *Grifola*, *Hericium*, *Lentinus* (*Lentinula*), *Pleurotus*, *Trametes* (*Coriolus*), *Schizophyllum*, and *Tremella* have been demonstrated to possess significant medicinal properties (Wasser, 1995; Ooi and Lui, 2002). *Pleurotus eryngii*, *Lyophyllum shimeji*, *Flammulina velutipes* and *Grifola frondosa* are edible mushrooms that have all been found to contain medicinally active compounds beneficial to health. However, many edible species of potent medicinal mushrooms such as *Ganoderma lucidum*, *Trametes versicolor* and *Inonotus obliquus* are very bitter and/or hard to eat and are used in the form of an extract, tea or powder (Wasser, 2002; Ajith and Janardhana, 2007). Of edible mushroom species, *Agaricus* represents the leader in world production, whilst in non-edible medicinal species, *Ganoderma*, which

belongs to the polypores[1] is the leader in terms of production. Several mushroom species belonging to the Polyporaceae family are now being regarded as the next candidate producers of valuable medicines (Mizuno, 1995).

The spectrum of detected pharmacological activities of Basidiomycetes is very broad, among their biological effects, the antifungal, anti-inflammatory, antitumor, antiviral, antibacterial, antiparasitic, immunomodulating, and hepatoprotective activities, are worthy to be mentioned; equally promising is their role in the regulation of blood pressure, as well as in the cure of cardiovascular disorders, in hypercholesterolemia and diabetes (Wasser and Weis, 1999b). Fungi are of special interest because like us they are eucaryotes and our metabolism is more related to them than to that of the prokaryotic bacteria (Pujol et al., 1990; Clericuzio et al., 2004). Even among the known species, the proportion of well investigated mushrooms is very low. This fact together with the knowledge about the great potential of microscopic fungi for production of bioactive metabolites [e.g. *Penicillium*, *Aspergillus*, *Tolypocladium inflatum* W. Gams, *Claviceps purpurea* (Fr.) Tul.], the experience in ethnomedicinal use of mushrooms, the ecologic need for fungi to produce bioactive secondary metabolites and the improved possibilities for genetic, pharmacological and chemical analysis let us assume that mushrooms have a great potential for successful bioprospecting (Lindequist et al., 2005).

1.4. Basidiomycetes as a source of phytochemicals /nutraceuticals

There are many species of Fungi from the division Basidiomycetes (mushrooms) that have been found to contain medicinally active compounds, which has been of great interest recently (Haak-Frendscho et al., 1993; Gan et al., 1998; Eo et al., 1999; Mattila et al., 2000; Hatvani, 2001; Wasser, 2002; Borchers et al., 2004; Zaidman et al., 2005). 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 polysaccharides with antitumor and

[1]Large group of terrestrial fungi of the phylum Basidiomycota (Basidiomycetes), which along with certain Ascomycota are the major source of pharmacologically active substances. They are constituted by pores that hold reproductive spores, rather than gills (Zjawiony, 2004).

immunostimulating properties. Many, if not all, Basidiomycetes mushrooms contain biologically active polysaccharides in fruit bodies, cultured mycelium, or culture broth (Wasser, 2002). Some advantages of using mushrooms over plants as sources of bioactive compounds are that often the fruiting body can be produced in much less time, the mycelium may also be rapidly produced in liquid culture that can be manipulated to produce optimal quantities of active products, or from mycelial biomass and supernatant of submerged cultured using bioreactors (Bose, 1955; Hikino et al ., 1985; Harttig et al., 1990; Sarkar et al ., 1993; Cui and Chisti, 2003).

Mushrooms contain compounds known as long-chain, large-molecular weight polysaccharides which when present in specific configurations or linkages (beta, 1-3 glucan and beta, 1-6 glucan) have strong effects on the immune system of humans (Mattila et al., 2000; Borchers et al., 2004; Quang et al., 2006). These natural compounds which cannot be artificially synthesized have proven useful in the treatment of both immune system deficiencies and also auto-immune disorders such as allergies, asthma and diabetes. Regardless of whether the pathology of the immune system is negative or positive (i.e., too weak or too strong), mushrooms have a beneficial regulating effect on the immune system (Poucheret et al., 2006). Data on mushroom polysaccharides, with most belonging to the group of β-glucans, have been collected from 651 species and 7 infraspecific taxa from 182 genera of higher Hetero- and Homobasidiomycetes (Wasser, 2002). Mushrooms also contain a variety of other complex secondary metabolites compounds such as phenolic compound, polyketides, triterpenoids and steroids which are specific to each mushroom and have specific effects in humans (Mattila et al., 2000; Wasser, 2002; Cheung et al., 2003; Borchers et al., 2004; Zaidman et al., 2005). These compounds have been used in the treatment of sexual impotence and dysfunction, high cholesterol levels, high blood pressure, high-altitude sickness, neurological disorders, and many other health problems (Paterson, 2006).

Several phytochemicals have been isolated from medicinal mushrooms and three of these, which are carcinostatic polysaccharide drugs, have been developed from mushrooms in Japan. These are “Krestin” (PSK), from the cultured mycelium of Kawaratake (*Trametes versicolor*), “Lentinan” from the fruiting bodies of Shiitake (*Lentinus edodes*) and “Schizophyllan” (Sonifilan) from the culture fluid of Suehirotake (*Schizophyllum commune*) (Mizuno, 1993). Lentinan and schizophyllan are pure β-glucans, whereas PSK is a protein bound polysaccharide (Larone, 2002). The biological

activity of these three products is related to their immunomodulating properties, which enhance the host's defence against various forms of infectious disease. These immunopotentiators, or immunoinitiators, are also referred to as "biological response modifiers" (Zjawiony, 2004; Zaidman et al., 2005).

1.4.1. Basidiomycetes as a source of antioxidants

The antioxidants in the human diet are of great interest as possible protective agents to help human body reduce oxidative damage. Recently, 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 (Ramarathnam et al., 1995).

Mushrooms have become attractive as a source of antioxidant nutraceuticals, as established in different *in vitro* assays. Some mushrooms species such as *Boletus badisus*, *Lepista nuda*, *Polyporus squamosus*, *Russula delica* or *Verpa conica* have been shown to possess higher chelating effects than synthetic antioxidants, like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), and α-tocoferol, while *Polyporus squamosus* and *Agaricus bisporus* showed higher chelating abillity than BHA and α-tocopherol. The reducing power of *Agaricus bisporus*, *Boletus badisus*, *Lepista nuda*, *Pleurotus ostreatus*, and *Polyporus squamosus* was also shown to be higher than BHA, BHT, and α-tocoferol (Elmaster et al., 2007). *Lentinus edodes*, *Volvariella volvacea* and *Agrocybe aegerita* extracts demonstrated antioxidant activity against lipid peroxidation of rat brain homogenate. *V. volvacea* was found to have comparable antioxidant activity to caffeic acid against the oxidation of human low-density lipoprotein (LDL) (Cheung and Cheung, 2005; Lo and Cheung, 2005). *Lentinus edodes* demonstrated higher capacity to inhibit hemolysis of erythrocytes than natural products isolated from Chinese herbs: luteolin-7-glucuronide-6′-methyl ester, rutin, 4′-demethyldeoxypo- dophyllotoxin, erianin and tanshinone I (Cheung et al., 2003). Among higher fungi, some species clearly demonstrate higher antioxidant properties when compared to others (Zhou et al., 1989; Zhou et al., 1991; Zhu et al., 1999). Among such mushroom species is included the tree oyster mushroom, *Pleurotus ostreatus* (Jacq.) P. Kumm, which possesses basic antioxidant compounds such as ascorbic acid, tocopherol, and β-carotene, as well as a high content in phenolic compounds (Yang et al., 2002).

Agaricus bisporus, *Agaricus arvensis*, *Boletus edulis*, *Cantharellus cibarius*, *Lactarius piperatus*, *Hygrocybe* sp. and *Pleurotus* sp. are some wild mushrooms species that contain high content of vitamine C (Cağlarirmak et al., 2002). L-ascorbic acid is the main biologically active form of vitamin C and is a potential antioxidant. It has the capacity to eliminate several different reactive oxygen species, keeps the membrane-bound antioxidant α-tocopherol in the reduced state and acts as a cofactor maintaining the activity of a number of enzymes, by keeping metal ions in the reduced state (Davey et al., 2000).

It has been reported that the antioxidant activity of plant materials is well correlated with their content of phenolic compounds (Velioglu et al., 1998). Indeed, phenolic compounds with major antioxidant abilities are heavily investigated for their potential high benefit on human health. This kind of metabolites start to be discovered in fungi kingdom and their antioxidative and free radical scavenging properties in some common edible mushrooms have been reported (Cheung et al., 2003; Mau et al., 2004; Lo and Cheung, 2005; Choi et al., 2006). Some phenolic components of wild mushrooms have already been identified; thus, *p*-hydroxybenzoic acid was found in *Amanita rubescens*, *Tricholoma equestre*, and *Russula cyanoxantha* (Ribeiro et al., 2006), and the presence of caffeic acid, *p*-coumaric acid, chlorogenic acid and rutin was also reported in *Cantharellus cibarius* (Valentão *et al.*, 2005). So, the effect of the phenolic content on the antioxidant activity of mushroom extracts should also be considered.

Indeed mushrooms are a sizeable source of antioxidant components already known or to be discovered. In addition, on a dietary as well as on a medical point of view, higher fungi may provide potent beneficial effects on human health either directly as antioxidant or through prevention of alterations underlying major pathological states such as cancer, diabetes, neurodegenerative diseases, cardiovascular diseases and metabolic syndrome. Antioxidant properties combined with antitumoral and immunomodulating effects lead to this "mushroom characteristic" health sustaining effect that they are recognized for (Lo and Cheung, 2005; Zhou et al., 2005). It is estimated that 50% of annual 5 million metric tons of cultivated mushrooms contain functional or medicinal properties, which may be utilized as source of biologically and physiologically active substances (Cheung et al., 2003). Therefore, the need to classify antioxidants in mushrooms and the isolation and structural characterization of these active components is important.

1.4.2. Basidiomycetes as a source of antimicrobials

The prevalence of infectious diseases is becoming a world wide problem; antimicrobial drugs have long been used for prophylactic and therapeutic purposes, but the drug-resistant bacterial strains have creating serious treatment problems (Jong and Donovick, 1990; Mulligen et al., 1993; Davis, 1994; Lorenzen and Anke, 1998; Donadio et al., 2002b). Antimicrobial drug resistance is of major economic concern having an impact on physicians, patients, health care administrators, pharmaceutical producers and the public (McGowan, 2001). In addition, bacteria and fungal pathogens have complicated the treatment of infectious diseases (Diamond, 1993; Baratta et al., 1998). So the demands to renewed effort should be made to seek antimicrobial agents effective against pathogenic microorganisms resistant to current treatment (Jong and Donovick, 1990; Karaman et al., 2003; Turkoglu et al., 2007).

Plant extracts as antimicrobial agents have been investigated (Vlietinck et al., 1995; Dorman and Deans, 2000) and have been found to contain many biologically active components (Cowan, 1999). In addition to plants extracts, research is being performed on fungi for their ability to mobilize the body's humoral immunity and in turn prevent bacterial, viral, or fungal pathogens that are resistant to current therapeutic agents (Wasser and Weis, 1999b).

Fungi are well known for the production of important antibiotic compounds such as penicillin, however, the occurrence of antibiotics in the class of Basidiomycetes (the mushrooms) is less well documented (Miles and Chang, 1997) and there are only few reviews that summarise the antibacterial activity within this division (Wasser and Weis, 1999b, Wasser and Weis 1999c; Zjawiony, 2004; Gao et al., 2005). Mushrooms need antibacterial and antifungal compounds to survive in their natural environment. It is therefore not surprising that antimicrobial compounds with more or less strong activities could be isolated from many mushrooms and that they could be of benefit for human (Lindequist et al., 1990).

The major philosophy of the search for antimicrobial activity from basidiomycetes is that humans (and animals) share common microbial pathogens with fungi, such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, so that we can benefit from defensive strategies used by fungi against microorganisms (Zjawiony, 2004). Some researchers have reported antibacterial and antifungal activity of several mushrooms (Lee et al., 1999; Hatvani, 2001; Kim and Fung, 2004; Gao et al., 2005;

Turkoglu et al., 2007), being both fruiting body and mycelium effect agents (Jong and Birmingham, 1993; Hatvani, 2001; Turkoglu et al., 2007).

There appear to be an increasing number of reports on Gram-positive bacteria developing resistance to virtually every clinically available drug (Donadio et al., 2002b), and basidiomycetous mushrooms have been shown to possess antibacterial activity against this group of bacteria. Early studies proposed that most antibacterial components from basidiomycetous fungi were potent against Gram-positive bacteria only (Robbins et al., 1947; Lee et al., 1982), but more recent work showed that extracts were also active against Gram-negative organisms *Proteus vulgaris* and *Escherichia coli*, *in vitro* (Yoon et al., 1994). The greater resistance of Gram-negative bacteria is most likely to be result of the cell walls consistence.

The mycelium of *Flammulina velutipes* was shown to have bacterial activity against the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* (Ishikawa et al., 2001). In addition to these two bacteria, the fruiting body extract from *Armillariella mellea* also exhibited activity against *B. cereus*, *in vitro* (Obuchi *et al*., 1990). Turkoglu et al. (2007) reported that *Laetiporus sulphurous* had a narrow antibacterial spectrum against Gram-negative bacteria and a strong inhibition of Gram-positive bacteria, including *Bacillus cereus*, *Bacillus subtilis*, *Micrococcus luteus* and *Micrococcus flavus*. Some *Agaricus* species (*A. campestis*, *A. bisporus* and *A. arvensis*) have been shown to produce compounds active against both Gram-positive and Gram-negative bacteria (Wasser and Weis, 1999c). Extracts from *Lentinus edodes* have also been shown to be active against both types of bacteria (Hirasawa et al., 1999), whilst *L. edodes* culture fluid only proved to be efficient with Gram-positive bacteria (*Bacillus cereus*, *Staphylococcus aureus* and *Staphylococcus pyogenes*) (Hatvani, 2001). Overall, extracts from mushrooms are observed to be more active against Gram-positive bacteria than Gram-negative bacteria (Smania et al., 1999).

Mushrooms are rich sources of natural antibiotics; in these, the cell wall glucans are well-known for their immunomodulatory properties, and many of the externalized secondary metabolites (extracellular secretions by the mycelium) combat bacteria (Benedict and Brady, 1972; Kupra et al., 1979) and viruses (Suzuki et al., 1990; Collins and Ng, 1997; Eo et al., 1999; Brandt and Piraino, 2000). However, only compounds from microscopic fungi are on the market as antibiotics till now, in spite of several compounds extracted from mushroom revealed antibacterial activity (Morita and

Kobayashi, 1967; Yasumoto et al., 1971), namely against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* (Takazawa et al., 1982).

There have been reports showing that triterpenes and sesquiterpenes are great antibacterial agents found in mushrooms (Donnelly et al., 1985; Pinducciu et al., 1995; Ishikawa et al., 2001; Wilkens et al., 2002). Armillaric acid, a terpenoid isolated from *A. mellea*, inhibits Gram-positive bacteria (Obuchi et al., 1990). Applanoxidic acid A, isolated from *Ganoderma annulare* (Fr.) Gilbn., shows weak antifungal activity against *Trichophyton mentagrophytes* (Smania et al., 2003). Steroids like 5a-ergosta- 7,22-dien-3b-ol or 5,8-epidioxy-5a,8a-ergosta-6,22-dien-3b-ol, isolated from *Ganoderma applanatum* (Pers.) Pat., proved to be weakly active against a number of Gram-positive and Gram-negative microorganisms (Smania et al., 1999). The antimicrobial activity of *Podaxis pistillaris* (L.: Pers.) Morse, used in some parts of Yemen for the treatment of 'nappy rash' of babies and in South Africa against sun burn, is caused by epicorazins. These substances belong to the group of epipolythiopiperazine-2,5-diones, an important class of biologically active fungal metabolites (Al-Fatimi, 2001). A polysaccharide Schizophyllan from *Schizophyllum commune* exhibited protective effects against *S. aureus*, *E. coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* infections in mice (Komatsu et al., 1973) and against an infection in yellowtail fish caused by a *Streptococcus* species (Matsuyama et al., 1992). The antibiotics, Psalliotin and Coriolin, from *Agaricus xanthoderma* and *Trametes* sp., respectively, have also been shown to inhibit Gram-positive bacteria (Takeuchi, 1969; Dornberger et al., 1986).

The importance of the Chinese Shiitake mushroom (*Lentinus edodes*) is well-known; besides its anti-tumour activity, it has been demonstrated to increase the host resistance to bacterial and viral infections (Jong and Birmingham, 1993). Lentinan and Krestin, from the cultivated fruiting body of *L. edodes* are polysaccharides that have exhibited antibacterial activity (Jong and Birmingham, 1993). Oxalic acid is one agent responsible for the antimicrobial effect of *L. edodes* (Berk.) Pegler against *S. aureus* and other bacteria (Bender et al., 2003). Ethanolic mycelial extracts from *L. edodes* also possess antiprotozoal activity against *Paramecium caudatum* (Badalyan, 2004).

The Fungi kingdom is thought to have weak antifungal activities (Mizuno, 1995) and, therefore, fungi have rarely been investigated for their bioactivity as antifungal agents. It is only recently that they have become of interest due to their secondary metabolites exhibiting a wide range of antimicrobial activities (Zjawiony, 2004; Lindequist et al., 2005). Nakajima et al. (1976) isolated an antifungal isocoumarin oospolactone, a

secondary metabolite, from *Gleophyllum sepiarium*, this compound was most active against the asexual ascomycete *Alternaria*. Abate and Abraham (1994) isolated antimicrobial sesquiterpenes, desoxyhypnophilin and hypnophilin from *Lentinus crinitus* that were not only active against Gram-positive bacteria *Bacillus cereus*, but also against the fungus spores of *Aspergillus niger*, *Aspergillus flavus* and *Mucor rouxii*. Interestingly, Anke and Sterner (1991) observed that derivatisation of sesquiterpenes to less polar compounds increased the antimicrobial and cytotoxic affects. Anke et al. (1980) isolated an antibiotic from the submerged culture of the mushroom *Marasmius scorodonius* (Fr.) Fr., which exhibited activity against bacteria, yeasts and filamentous fungi. Later on, Ishikawa et al. (2001) isolated two cuparene-type sesquiterpenes from the culture medium of *F. velutipes*, and observed both to have antifungal activity against the fungus *Cladosporium herbarum*. Hatvani (2001) tested the culture fluid of the mushroom *L. edodes* and found that it exhibited poor activity against the yeast *Candida albicans*. Antifungal compounds have also been found in the extracts of *L. edodes* (Takazawa et al., 1982). Antifungal drugs available today are not always successful in treating immunocompromised patients due to the ineffectiveness or toxicity that many of them have on the host (Seltrennikoff, 1995), and hence, there is a need for the identification of novel antifungal agents. Overall, mushrooms have a great potential for the production of useful bioactive metabolites being prolific resource for drugs.

1.5. Basidiomycetes as sources of nutrients

Since human civilization, fruiting bodies of mushrooms are appreciated not only for texture and flavour but also for their chemical and nutritional properties (Manzi et al., 1999). More than 140,000 species of mushrooms exist in nature, but less than 25 species (*Agaricus bisporus*, *Pleurotus* spp., *Lentinus edodes*, *Volvariella volvacea*, *Auricularia* spp. etc.) are widely accepted as food and only a few have attained the level of an item of commerce (Smith, 1972; Lindequist et al., 2005). However, wild edible mushrooms have been used as food and food flavouring material in soups for centuries, due to their unique and delicate flavour (Cheung and Cheung, 2005) and they have also been traditionally eaten seasonally by specific groups of people (local people, enthusiasts and gourmets). Nevertheless, wild mushrooms are becoming more and more important in our diet not only for their nutritional (Breene, 1990; Manzi et al., 1999) and

organoleptic (Maga, 1981) values, but also for their pharmacological characteristics (Bobek et al., 1991; Bobek et al., 1995; Bobek and Galbavy, 1999), as referred to in section 1.3. Wild edible mushrooms are rich in trace minerals, and have high water, protein, fibre, carbohydrate contents and low fat/energy levels making them an excellent food for use in low caloric diets (Ogundana and Fagade, 1982; Senatore, 1988; Aletor, 1995; Fasidi, 1996; Longvah and Deosthale, 1998; Thimmel and Kluthe, 1998; Yildiz et al., 1998; Díez and Alvarez, 2001; Agahar-Murugkar and Subbulakshmi, 2005). Edible mushrooms species are highly nutritious and may compare favourably with meat, eggs and milk. Some investigations have even contended that the amino acid compositions of mushrooms are comparable to animal proteins (Fink and Hoppenhaus, 1958; Gruen and Wong, 1982), which is particularly important considering the cost of of those proteins and the outbreak of diseases connected with animal meat. Although the nutritional potential or implications of this gradual replacement of meat with mushroom requires careful examination, which involves detailed chemical and biological studies (Aletor, 1995). Mushrooms also appear to be a good source of vitamins, such as vitamin B (thiamine-B_1, riboflavin-B_2), vitamin C (ascorbic acid) and vitamin E (tocopherol). Mushrooms are deficient in vitamin D, however they are found to be a rich source of ergosterol, the precursor of vitamin D_2 (Breene, 1990).

Carbohydrates were found in wild mushrooms in relevant amounts, representing about 3-65% of dry weight (Manzi et al., 1999; Manzi, et al., 2001; Sanmee et al., 2003; Cheung and Cheung, 2005). Wild edible mushrooms are rich in non-starch polysacaccharides (dietary fiber, 3-32% of dry weight), while monosaccharides and disaccharides are usually present in lower concentration (Manzi and Pizzoferrato, 2000; Pizzoferrato et al., 2000; Wannet et al., 2000). Glycogen, monosaccharides and disaccharides (such as trehalose) and sugar alcohols (such as mannitol) are common carbohydrates found in wild mushrooms-(Harada et al., 2004). As mentioned earlier, β-glucan is a structural polysaccharide that is found abundantly in edible mushrooms. The insoluble fraction chitin, which is structural polymer of the fungal cell, also exists significantly in mushrooms (Manzi et al., 2001).

The proteins content in mushrooms is usually very high in majority of the wild mushrooms, representing 10-44% of dry weight (Longvah and Deothale, 1998). They are also proven to be a good source of essential amino acids such as: leucine, valine, threonine, lysine, methionine and tryptophan. Leucine and valine were found to be the most abundant essential amino acids, comprising 25-40% of the total amino acid

content (Chang et al., 2001; Yang et al., 2001; Mdachi et al., 2004; Guo et al., 2007). Wild mushroom proteins also contain considerable amounts of non-essential amino acids such as: alanine, arginine, glycine, glutamic acid, aspartic acid, proline and serine. They are important in providing structure to cells, tissues and organs and therefore essential for growth and repair (León-Guzmán et al., 1997).

The wide variety and abundance of minerals are the most characteristic features of fungi; they are a good source of potassium, magnesium, calcium, copper, iron, zinc, and phosphorus and have low content in sodium. Generally, concentrations of minerals in mushrooms are higher than in agricultural plants, vegetables and fruits (Manzi et al., 2001).

Fresh mushrooms are an excellent choice for low energy diets, as they have high water and low fat content (average of 2-8% of dry weight). Fat in mushrooms contains all classes of lipid compounds including free fatty acids, mono-, di-, and triglycerids, sterols, sterol esters and phospholipids (Manzi et al., 1999). Many wild edible mushrooms species are known to store relatively high levels of polyunsaturated fatty acids such as palmitic, stearic and linoleic acid, being linoleic acid the major essential fatty acid predominant in most edible wild species. Linoleic acid is the precursor of 1-octen-3-ol, known as the alcohol of fungi, which is the principal aromatic compound in most fungi species and contribute to mushrooms flavour (Maga, 1981). Linoleic acid or oleic acid ratios could constitute important parameters for the taxonomical difference between species of the same genus (Díez and Avarez, 2001).

1.6. Aims of investigation

Mushrooms collection and consumption is a traditional practice in some Portuguese regions like Trás-os-Montes, Beiras and Alentejo. In most of the cases, mushrooms collection is for self-consumption, despite the risks of this action. Nevertheless, in the last years the collection of wild mushrooms for commercialisation became intensified due to national and international interests. Particularly, the Northeast of Portugal due to climatic conditions and flora diversity is one of the European regions with higher wild edible mushrooms diversity, some of them with great gastronomic relevance. This fact stimulates the commercialisation from Trás-os-Montes to foreign countries like Spain, France and Italy.

Due to the great intensity of mushrooms harvest in some regions of Portugal, it is important to stimulate research in mycology area. In fact, mushrooms have become attractive as functional foods and as sources for the development of drugs and nutraceuticals. In addition to dried mushrooms, alternative or substitute mushroom products are mycelia that could also be used as food and food-flavouring material, for the production of nutraceuticals, or in the formulation of functional foods. The add-value arising from mushrooms/mycelia bioactive properties can leave to an increase in its consumption and, therefore, stimulating the commercialisation of local edible species and the *in vitro* production of mycelium mushroom (e.g. for pharmaceutical industry). This natural resource may be an economic alternative for the farmers from the Northeast of Portugal known for being an ill-favoured region. Mushroom production and harvest by rural population may also stimulate the conservation industry. Also, the *in vitro* production of mycelium or other by-products can be improved for local biotechnologists. Mushrooms can also have social, educative and recreation interests, and its search may stimulate the well-known "micotourism", namely for the city population that can go to the country following mushrooms harvest routes according to their nutritional value.

Most of the mushrooms consumers do not know their nutritional composition or their medicinal properties. The nutritional and phytochemical value and taste components of mushrooms/mycelia have to be determined and divulged to the population. Several local species have been reported for their medicinal activity, such as *Leucopaxillus* species used in pharmaceutical industry for extraction of clitocybin antibiotic. Other mushrooms are ectomycorrhizal (associations between fungi and some plant roots), bringing great benefits to the host plant, namely the increase in its growth, nutrients and water absorption, as well as a higher tolerance to pathogens, heavy metals and to extreme temperatures. Mushrooms study can contribute for the socio-economic development by showing the product relevance, which brings a higher value to this natural source and develops the valorisation of its products such as mycelium and functional components. Besides the increase of mushrooms economic value we also intend with this research to stimulate the creation of rules for their production, commercialisation, and contribute to human health.

The aims of this investigation are to evaluate:

1) Bioactive/functional properties of Portuguese wild mushrooms and their mycelia, produced by *in vitro* cultures, namely i) antioxidant activity through chemical (reducing

power; scavenging effects on DPPH radicals; inhibition of peroxidation in liposome solutions) and biochemical assays (inhibition of erythrocyte hemolysis mediated by peroxyl free radicals; inhibition of lipid peroxidation using brain tissues), and ii) antimicrobial activity using collection microorganisms or clinical isolates (Gram-positive and Gram-negative bacteria, and fungi).

2) Nutritional value and chemical composition: contents in water, ash, proteins, fats and carbohydrates; individual profiles in sugars and fatty acids.

3) Compounds related to bioactive properties, such as phenolic compounds, tocopherols, ascorbic acid and carotenoids.

CHAPTER II

LITERATURE REVIEW

2.1. Phytochemicals/nutraceuticals

There is emerging interest in the use of naturally occurring antioxidants for the preservation of foods and in the management of a number of pathophysiological conditions, most of which involve free radical damage. The implication of oxidative and nitrosative stress in the etiology and progression of several acute and chronic clinical disorders has led to the suggestion that antioxidants can have health benefits as prophylactic agents (Sobrattee et al., 2005). Epidemiological studies have consistently shown that a high dietary intake of fruits and vegetables is strongly associated with reduced risk of developing chronic diseases, such as cancer and cardiovascular disease (Willett, 1994; Temple, 2000; Willett, 2002; Liu, 2004; Sobrattee et al., 2005). This suggests that change in dietary behaviour, increasing consumption of plant-based foods, which contain significant amounts of bioactive phytochemicals, may provide desirable health benefits beyond basic nutrition to reduce the risk of chronic diseases (Liu, 2003; Marchand, 2002).

The number of individual phytochemicals already identified in fruits and vegetables is estimated in >5,000, but a large percentage still remain unknown and need to be identified before we can fully understand the health benefits of phytochemicals in whole foods (Liu, 2004). However, more and more convincing evidence suggests that the benefits of phytochemicals in fruits and vegetables may be even greater than is currently understood, because the oxidative stress induced by free radicals is involved in the etiology of a wide range of chronic diseases (Ames and Gold, 1991). Phytochemicals can be classified as carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds (**Figure 2.1.1**) (Liu, 2004).

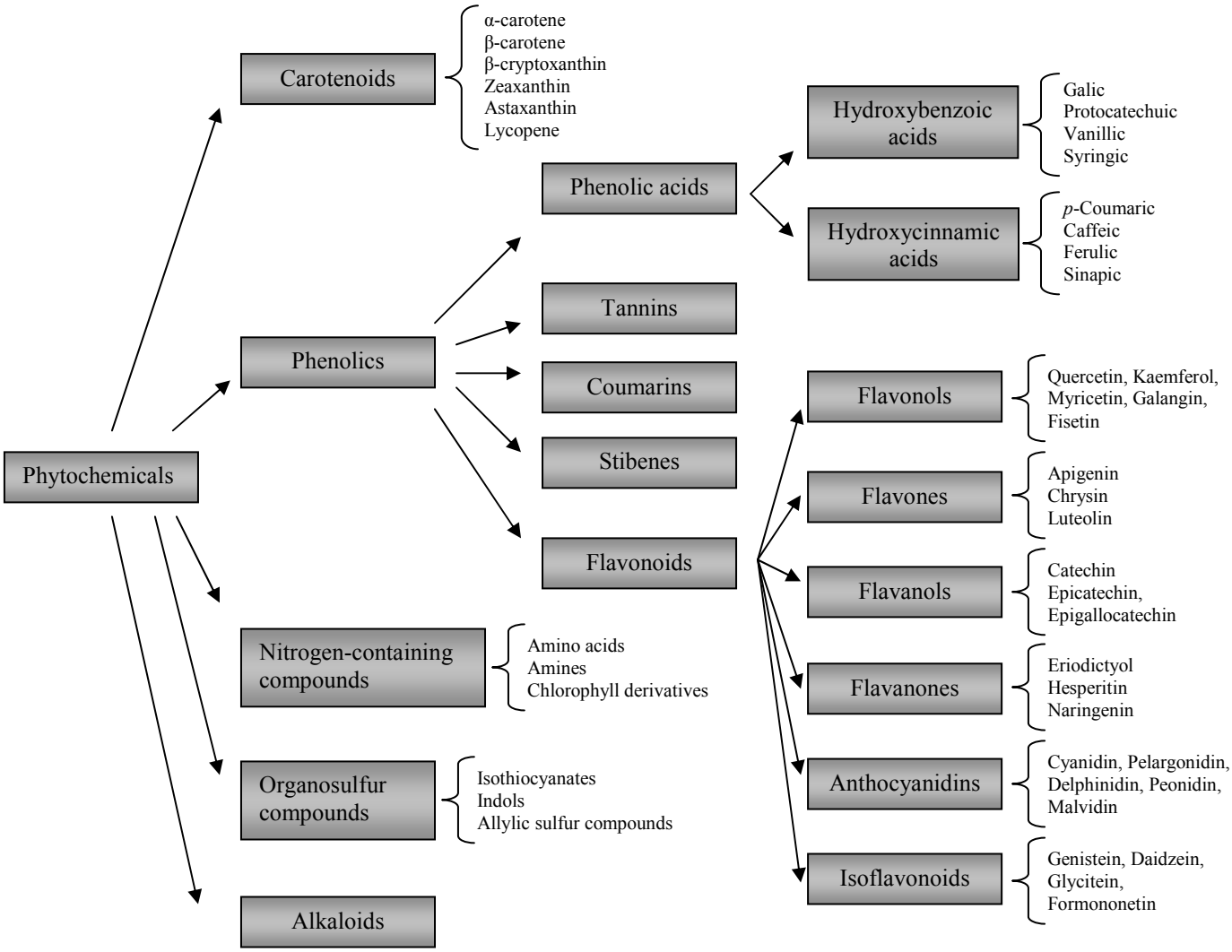


Figure 2.1.1. Classification of dietary phytochemicals.

The most studied of the phytochemicals that are believed to have antioxidant properties, have been the phenolic compounds, vitamins C and E, and carotenoids.

The term "nutraceutical" was coined in 1989 by the Foundation for Innovation in Medicine (New York, US), to provide a name for this rapidly growing area of biomedical research. A nutraceutical was defined as any substance that may be considered a food or part of a food and provides medical or health benefits including the prevention and treatment of disease (DeFelice, 1992). Nutraceuticals may range from isolated compounds or dietary supplements to genetically engineered "designer" foods, plant extracts or processed products such as cereals, soups and beverages. Some compounds/products recognised as nutraceuticals are indicated in the **Table 2.1.1**.

Table 2.1.1. Selected nutritive nutraceuticals

Dietary fiber
Polyunsaturated fatty acids (PUFA, fish oil)
Proteins, peptides, amino acids, keto acids
Minerals
Antioxidative vitamins
Other antioxidants (glutathione, selenium, etc.)

2.1.1. Phenolic compounds

Phenolic compounds are aromatic hydroxylated compounds, possessing one or more aromatic rings with one or more hydroxyl groups, being commonly found in vegetables, fruits and many food sources that form a significant portion of our diet, and some of which are among the most potent and therapeutically useful bioactive substances. Phenolic derivatives represent the largest group of 'secondary plant products' synthesized by higher plants, probably as a result of antioxidative strategies adapted in evolution by respirative organisms starting from precursors of cyanobacteria (Bennick, 2002; Apak et al., 2007). Natural phenolic compounds accumulate as end-products from the shikimate and acetate pathways (Carey, 2003) and can range from relatively simple molecules (phenolic acids, phenylpropanoids, flavonoids) to highly polymerised compounds (lignins, melanins, tannins), with flavonoids representing the most common and widely distributed sub-group (Bravo et al., 1998). Currently, more than 8,000 distinct flavonoids have been identified (Andersen and Markam, 2006). Many foods that are considered as healthy contain relatively high levels of flavonoids (Escarpa and Gonzalez, 2001).

Phenolic compounds have specific health effects, even though they are non-nutritive compounds. In our diet they might provide health benefits associated with reduced risk of chronic diseases that may be due to their ability to reduce agents by donating hydrogen and quenching singlet oxygen (Nijveldt et al., 2001). Antioxidant properties of phenolic compounds also play a vital role in the stability of food products, as well as in the antioxidative defense mechanisms of biological systems (Macheix and Fleuriert, 1998).

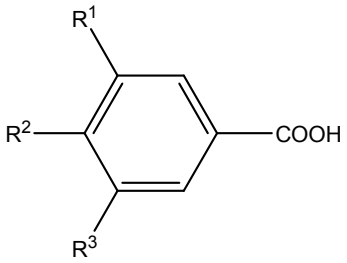
The overall effectiveness of a natural phenolic antioxidant depends on the involvement of the phenolic hydrogen in radical reactions, the stability of the natural antioxidant radical formed during radical reactions, and the chemical substitutions present on the structure (Hall, 2001). The substitutions on the structure are probably the most significant with respect to the ability of a natural antioxidant to participate in the control of radical reactions and to form resonance-stabilized natural antioxidant radicals (Barlow, 1990). The role of phenolic antioxidants (ArOH) is to interrupt the chain reaction according to:

$$RO_2^{\bullet} + ArOH \longrightarrow ROOH + ArO^{\bullet}$$

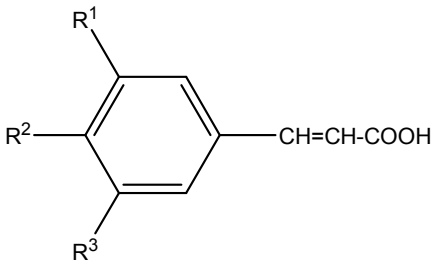
To be effective $ArO^{\bullet}$ must be a relatively stable free radical, so that it reacts slowly with substrate RH but rapidly with $RO_2^{\bullet}$, hence the term "chain-breaking antioxidant" (Wrigth et al., 2001).

2.1.1.1. Phenolic acids

Phenolic acids can be divided into two major groups, hydroxybenzoic acids and hydroxycinnamic acids (**Figure 2.1.2**), which are derived from non-phenolic molecules benzoic and cinnamic acid, respectively (Harborne and Williams, 2000).



Hydroxybenzoic acids



Cinnamic acids derivatives

	Substitution		
	R^1	R^2	R^3
p-Hydroxibenzoic	H	OH	H
Protocatechuic	H	OH	OH
Vanillic	CH_3O	OH	H
Syringic	CH_3O	OH	CH_3O
Gallic	OH	OH	OH

Cinnamic acid derivatives	Substitution		
	R^1	R^2	R^3
p-Coumaric	H	OH	H
Caffeic	OH	OH	H
Ferulic	CH_3O	OH	H
Sinapic	CH_3O	OH	CH_3O

Figure 2.1.2. Chemical structure of the benzoic and cinnamic acids derivatives.

Hydroxybenzoic acid derivatives commonly occur in the bound form and are typically a component of a complex structure like lignins and hydrolyzable tannins. They can also be found linked to sugars or organic acids in plant foods. Hydroxycinnamic acid derivatives are mainly present in plants in the bound form, linked to cell-wall structural components, such as cellulose, lignin, and proteins, as well as associated to organic acids, such as tartaric or quinic acids (i.e., chlorogenic acids), through ester bonds (Liu, 2004).

The antioxidant activity of phenolic acids is due to the phenolic hydrogens (Naczk and Shahidi, 2004). Hydroxyl substitutions at *ortho* and *para* positions also will enhance antioxidant activity (Hall, 2001). Intramolecular hydrogen bonds are formed by *ortho* substituted phenols (*e.g.*, 1, 2-dihydroxybenzene) during radical reactions, which increases the stability of the phenoxy radical (Baum and Perun, 1962). It has been reported that caffeic acid was a better antioxidant than ferulic acid or *p*-coumaric acid (Hall, 2001). The second hydroxy group at position *ortho* allows the formation of intramolecular hydrogen bonds, which results in stronger antioxidant activity than those of compounds containing a methoxy (OCH_3) substitution *ortho* to the hydroxy group (Baum and Perun, 1962). Ferulic acid contains *ortho* methoxy substitution in its structure that may provide a stabilizing effect on the phenoxyl radical, which enhances its antioxidant activity over *p*-coumaric acid (Hall, 2001). The superior antioxidant activity of trihydroxybenzoic acid (*i.e.*, gallic acid) over 3, 4- dihydroxybenzoic acid (*i.e.*, protocatechuic acid) is due to the presence of three hydroxyl groups in trihydroxybenzoic acid.

The acid proton appears to have little impact on antioxidant activity. Both caffeic acid and chlorogenic acid, the resultant compound after replacement of the acid proton of caffeic acid with quinic acid via an ester bond, were equally effective in controlling lipid oxidation (Hall, 2001). The allylic group, as found in cinnamic acid derivatives, provides enhanced antioxidant activity when compared to benzoic acid derivatives. Pratt and Hudson (1990) reported that caffeic acid (3, 4- dihydroxycinnamic acid) was a better antioxidant than protocatechuic acid (3, 4- dihydroxybenzoic acid) in a lard system. The allylic group may improve the resonance stability of the phenoxyl radical (Cuvelier et al., 1992).

2.1.1.2. Flavonoids

Flavonoids represent a large group of phenolic compounds with antioxidant activity, that occur naturally in plants and are found in fruits, vegetables, grains, barks, roots, stems, flowers, and derived products like tea and wine. These compounds have been linked to reduce the risk of major chronic diseases (Blokhina et al., 2003, Liu, 2004). They are characterized for the carbon skeleton C6–C3–C6. The basic structure of these compounds consists of two aromatic rings (A and B rings) linked by a three carbon chain that is usually in an oxygenated heterocycle ring, or C ring (**Figure 2.1.3**) (Yanishlieva, 2001).

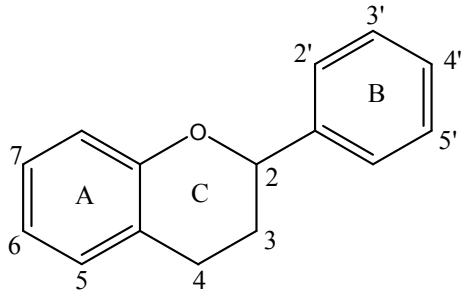


Figure 2.1.3. The generic structure of flavonoids

Several classes of flavonoids are delineated on the basis of differences in the generic structure of the heterocycle C ring and can be classify into flavonols, flavones, flavanols, flavanones, anthocyanins and isoflavonoids (**Figure 2.1.4**) (Nijveldt et al., 2001). Flavonols (quercetin, kaempferol, and myricetin), flavones (luteolin and apigenin), flavanols (catechin, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate), flavanones (naringenin), anthocyanidins, and isoflavonoids (genistein) are common flavonoids in the diet (Liu, 2004).

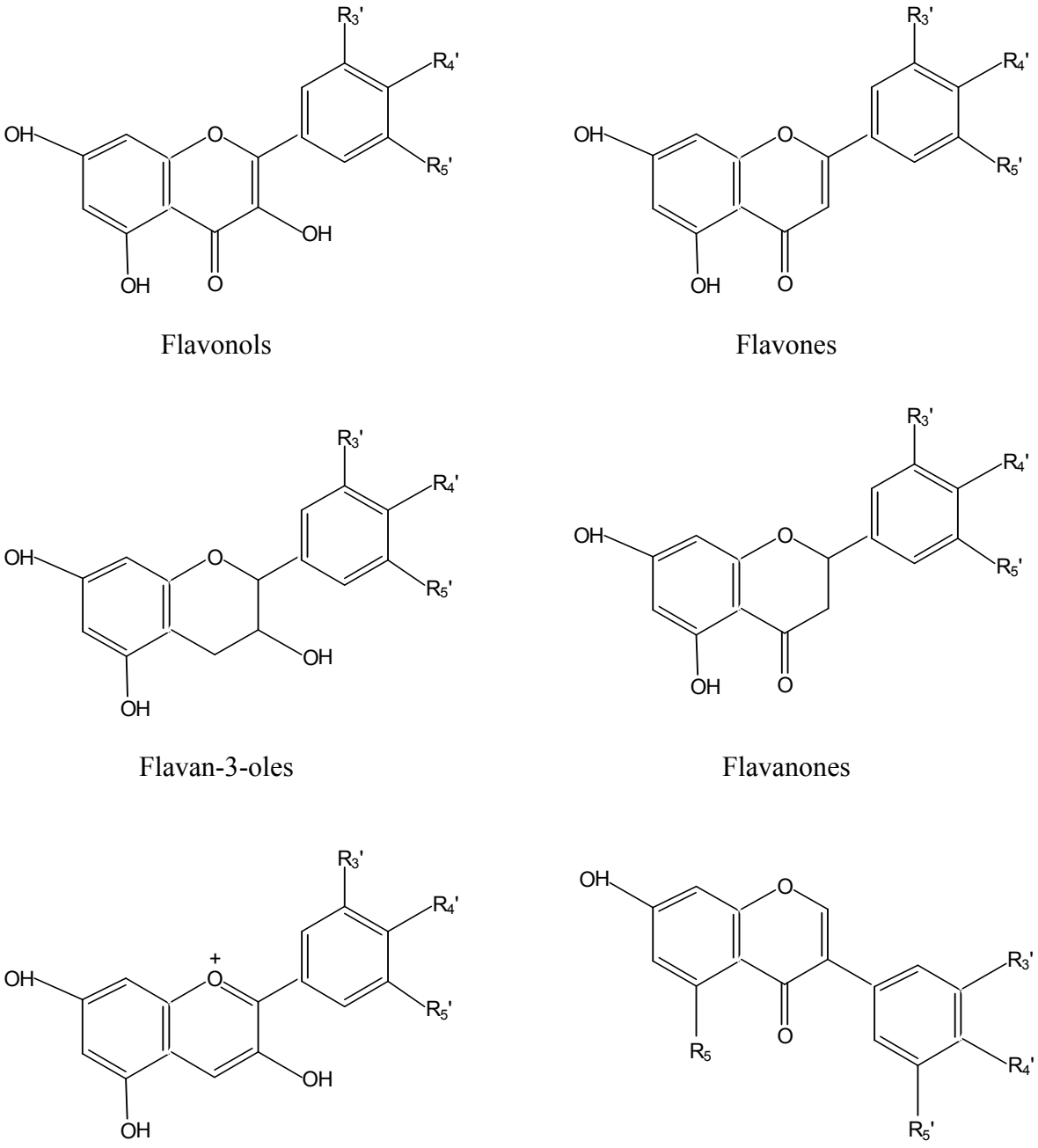


Figure 2.1.4. Chemical strutures of several flavonoids

Flavonoids are most frequently found in nature as conjugates in glycosylated or esterified forms but can also occur in food as aglycones, especially as a result of the effects of food processing (Hollman and Arts, 2000). Flavonols are the most abundant flavonoids in foods. Flavanones are mainly found in citrus fruit and flavones in celery.

Catechins are present in large amounts in green and black tea and in red wine, whereas anthocyanins are abundant in berries. Isoflavones are almost exclusively found in soy foods (Marchand, 2002).

Multiple mechanisms have been identified as involved in the health-promoting effects of flavonoids, including antioxidant, anti-inflammatory and anti-proliferative activities, inhibition of bioactivating enzymes, or induction of detoxifying enzymes (Marchand, 2002). The antioxidant property of flavonoids was the first mechanism of action studied, in particular with regard to their protective effect against cardiovascular diseases. Flavonoids have been shown to be highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and various free radicals (Bravo, 1998), which are possibly involved in DNA damage and tumor promotion (Cerutti, 1985).

2.1.1.3. Determination of total phenolics using the Folin and Ciocalteu reagent

This assay originally was designed and used for the analysis of protein by reaction between the reagent and tyrosine (which contains a phenol group) residues in proteins (Folin and Ciocalteu, 1927). The method was further adopted for the analysis of total phenols in many studies and, in fact, is now commonly known as the total phenols (or phenolics) assay. Molybdotungstate reagent oxidizes phenols and yields a coloured product with an absorption maximum at 745-750 nm. The total phenols assay actually measures the reducing capacity of a sample (Singleton et al., 1999). Numerous publications reported excellent linear correlations between "total phenolic profiles" as determined by the Folin and Ciocalteu (FC) assay and "the antioxidant capacity" (Huang et al., 2005).

The precise mechanism of the FC reaction is not yet well established. The reagent contains heteropolyphosphotungstates-molybdates that, under basic conditions, react with phenolic compounds to form a phenolate anion, possibly (phenol-$MoW_{11}O_{40}$)$^{4-}$, by dissociation of a phenolic proton. This sequence of reversible one- or two-electron reduction reactions leads to blue-coloured products. In essence, it is believed that the complex and electron transfer reaction between Mo (VI) and reductants reduces the molybdenum (Huang et al., 2005; Prior et al., 2005):

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

A number of papers have reported different reference standards for the total phenolics assay, including catechin equivalents (Katsube et al., 2003; Vinson et al., 2001), tannic acid equivalents (Nakamura et al., 2003), chlorogenic acid equivalents (Wang et al., 2003), caffeic acid equivalents (Maranz et al., 2003), protocatechuic acid equivalents (Cai et al., 2003), vanillic acid equivalents (Jayasinghe et al., 2003), gallic acid equivalents (Cheung et al. 2003) and ferulic acid equivalents (Velioglu et al., 1998).
The total phenolics assay has become a routine assay in studying phenolic antioxidants and it is simple and sensitive (Huang et al., 2005). However, a number of substances, particularly sugars, aromatic amines, sulfur dioxide, ascorbic acid and other enediols and reductones, organic acids, Fe(II) and Cu(I), interfere with the total phenolics method, so correction for interfering substances should be considered. In addition, reaction between the FC reagent and some nonphenolic organics, including adenine, adenosine, alanine, aniline, aminobenzoic acid, ascorbic acid, benzaldehyde, creatinine, cysteine, cytidine, cytosine, dimethyaniline, diphenylamine, ethylenediamine tetraacetic acid (EDTA), fructose, guanine, guanosine, glycine, histamine, histidine, indole, methylamine, nitriloacetic acid, oleic acid, phenylthiourea, proteins, pyridoxine, sucrose, sulfanilic acid, thiourea, thymine, thymidine, trimethylamine, tryptophan, uracil, uric acid and xanthine, may interfere with the assay. Also, reaction between FC reagent and some inorganic substances, such as hydrazine, hydroxyammonium chloride, iron ammonium sulfate, iron sulfate, manganese sulfate, potassium nitrite, sodium cyanide, sodium metabisulfite, sodium phosphate, sodium sulfite and tin chloride, may also lead to overestimated phenolics concentrations (Prior et al., 2005).

2.1.2. Vitamins

Vitamins have been defined as a group of naturally occurring organic substances present in small quantities in foodstuffs that cannot be synthesized in sufficient quantities by an organism, and must be obtained from the diet. These nutrients are essentials for the normal metabolism and well being of animals and man (Marks, 1988) and their lack in the diet causes deficiency diseases (Manan, 1994). A group of vitamins (vitamin E and vitamin C) with potent antioxidant activities have recently received a great deal of attention because of their action on immunity and disease etiology. Vitamin E (α-

tocopherol) and vitamin C (ascorbate) are naturally-occurring antioxidant nutrients that play important roles in health by inactivating harmful free radicals produced through normal cellular activity and from various stressors (Chen, 1993; Chew, 1995). Sato et al. (1990) suggested that water-soluble chain radicals, such as vitamin C, function as a primary defense against aqueous radicals, while vitamin E acts as lipophilic chain-breaking antioxidant and is responsible for scavenging lipid peroxyl radicals (Liu et al., 2008).

Vitamin E is a term frequently used to designate a family of chemically related compounds, namely tocopherols and tocotrienols, which share a common structure with a chromanol head and isoprenic side chain (Amaral et al., 2005). Vitamin E is composed of eight chemical compounds: α-, β-, γ- and δ- tocopherols and four corresponding tocotrienols (**Figure 2.1.5**) (Kamal-Eldin et al., 2000; Ryynanen et al., 2004). All four members of each series are neither isomers nor homologues; they differ in both the number of methyl substituents and their positions on the phenolic ring (Kamal-Eldin et al., 2000). Each form has its own biological activity, which is the measure of potency of functional use in the body (Traber and Packer, 1995). Vitamin E is an important natural antioxidant in foods, especially those rich in polyunsaturated fatty acids (Kamal-Eldin and Appelqvist, 1996; Blokhina et al., 2003). Due to its role as a scavenger of free radicals, vitamin E is also believed to protect our bodies against degenerative malfunctions, mainly cancer and cardiovascular diseases (Burton and Traber, 1990).

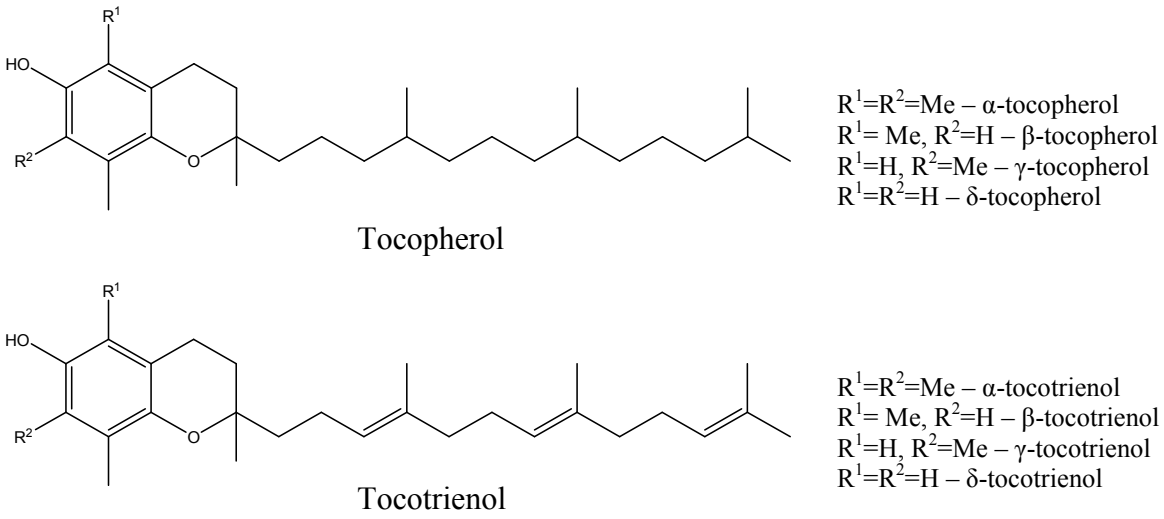


Figure 2.1.5. Chemical structure of the four vitamers of tocopherol and tocotrienol.

Vitamin E reacts with peroxyl radicals produced from polyunsaturated fatty acids in membrane phospholipids or lipoproteins to yield a stable lipid hydroperoxide. They act as antioxidants by donating a hydrogen atom to peroxyl radicals of unsaturated lipid molecules, forming a hydroperoxide and a tocopheroxyl radical, which reacts with other peroxyl or tocopheroxyl radicals forming more stable adducts (Lampi et al., 1999).

$$LH + \text{Oxidant initiator} \longrightarrow L^{\bullet}$$

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + \text{Tocopherol} \longrightarrow LOOH + \text{Tocopherol}^{\bullet}$$

In the past α-tocopherol was considered the most active form of vitamin E in humans and it was reported to exhibit the highest biological activity (Traber, 1999). However, many recent publications have been focusing on the health effects of the other vitamin E isoforms (Schwenke, 2002; Amaral et al., 2005; Sanagi et al., 2005).

Vitamin C also known as ascorbic acid is one of the simplest vitamins. It is related to the C6 sugars, being the aldono-1,4-lactone of a hexonic acid (L-galactonic or L-gulonic acid), and contains an enediol group on carbons 2 and 3 (**Figure 2.1.6**), that exists in two stereoisomeric forms: an L-isomer and a D-isomer (Margolis and Schapira, 1997; Davey et al., 2000). It is a metabolite with strong antioxidant activity and a cofactor for enzymes catalyzing numerous biochemical reactions, including those neutralizing the effects of reactive oxygen species (Giovannoni, 2007).

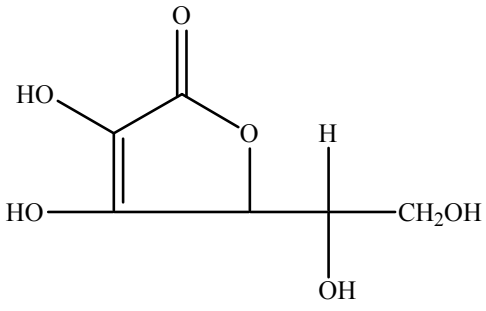


Figure 2.1.6. Chemical structure of ascorbic acid.

Vitamin C is a necessary nutrient for a limited number of animals, including humans, that are incapable of its synthesis and that must secure vitamin C by means of dietary

uptake (Hancock and Viola, 2005; Giovannoni, 2007). Plants produce large amounts of L-ascorbic acid to facilitate resistance to the oxidative stresses, they are also the primary source of vitamin C intake with respect to human diets (Hancock and Viola, 2005). In addition, ascorbic acid is thought to exert a protective role against various oxidative stress-related diseases such as heart disease, stroke, cancer, several neurodegenerative diseases and cataractogenesis (Halliwell, 1996). Vitamin C can protect biomembranes against lipid peroxidation damage by eliminating peroxyl radicals in the aqueous phase before the latter can initiate peroxidation (Davey, 2000). Vitamin C is effective against superoxide, hydroxyl radical, hydrogen peroxide, peroxyl radical and singlet oxygen (Sies et al., 1992).

Cooperative interactions exist among vitamin C and vitamin E. They interact synergistically at the membrane-cytosol interface to regenerate membrane-bound oxidized vitamin E (Li and Schellhorn, 2007). Vitamin E, in the form of α-tocopherol, is a key lipophilic antioxidant in human circulation and the vasculature and plays a role in many key processes contributing to the onset and progression of atherosclerosis (Kaliora et al., 2006). As a lipophilic antioxidant, vitamin E can interact with the lipid components in the vascular systems, notably LDL, and protects them from atherogenic oxidative modification (Burton and Ingold, 1986). Conversely, the lipid-bound α - tocopherols can be oxidized by aqueous-phase radicals and transformed into reactive tocopherol radicals, which, in turn, react with the unsaturated lipids of the lipoprotein, initiating lipid oxidation by a tocopherol-mediated peroxidation reaction (Bowry and Stocker, 1993; Ingold et al., 1993). Oxidized vitamin E can be reduced back to its antioxidant form by other aqueous-phase reductants, like ascorbic acid (Ingold et al., 1993).

Ascorbic acid reacts rapidly with the tocopherol radical by reducing the ascorbate radical (semidehydroascorbate) is to ascorbate by NADH-dependent semidehydroascorbate reductase.

$$\text{Ascobic acid} + \text{Vitamin E}^{\bullet} \longrightarrow \text{Ascorbate}^{\bullet} + \text{Vitamin E}$$

$$\text{Ascobate}^{\bullet} + \text{NADH} \longrightarrow \text{Ascorbate} + \text{NAD}^{\bullet}$$

As such, it may be conducive for vitamin E regeneration (Neuzil et al., 2001). In addition, ascorbate may sequester aqueous radicals in the plasma before they can oxidize vitamin E in the lipid phase and affords protection for lipid-bound tocopherols.

The interactions among these antioxidant nutrients are likely very important in protecting cells because the concentration of each antioxidant alone may not be adequate to effectively protect these cells against lipid peroxidation (Chew, 1995; Nagaoka et al., 2007).

2.1.3. Carotenoids

Carotenoids are nature's most widespread pigments and have also received substantial attention because of both their provitamin and antioxidant roles (Liu, 2004). Carotenoids are synthesized by plants and microorganisms but not animals. Fruits and vegetables constitute the major sources of carotenoid in human diet (Mangels et al., 1993; Agarwal and Rao, 2000; Johnson, 2002). They are present as micro-components in fruits and vegetables and are responsible for their yellow, orange and red colors. More than 600 carotenoids have so far been identified in nature. However, only about 40 are present in a typical human diet. Of these 40 about 20 carotenoids have been identified in human blood and tissues. Close to 90% of the carotenoids in the diet and human body is represented by β -carotein, α -carotein, lycopene, lutein and β-cryptoxanthin (Gerster, 1997).

All carotenoids posses a 40-carbon skeleton of polyisoprenoid structure, a long conjugated double bonds chain of forming the central part of the molecule and a near bilateral symmetry around the central double bond, as common chemical features (Britton, 1995). This gives them their shape, chemical reactivity, and light-absorbing properties. Different carotenoids are derived essentially by modifications in the base structure by cyclization of the end groups and by introduction of oxygen-containing functional groups, giving them their characteristic colors and antioxidant properties. Lycopene and β-carotene are examples of acyclized and cyclized carotenoids, respectively. Structures of some common carotenoids are shown in **Figure 2.1.7**. Due to the presence of the conjugated double bonds, carotenoids can undergo isomerization to *cis-trans* isomers. Although the *trans* isomers are more common in foods and are more stable, very little is known about the biological significance of carotenoid isomerization in human health (Rao and Rao, 2007). Carotenoids are thought to be responsible for the beneficial properties in preventing human diseases including cardiovascular diseases, cancer and other chronic diseases (Paiva and Russell, 1999; Astrog et al., 1997). They are important dietary sources of vitamin A (Paiva and Russell, 1999), being β-Carotene,

α-carotene, and β-cryptoxanthin able to function as provitamin A (Liu, 2004).

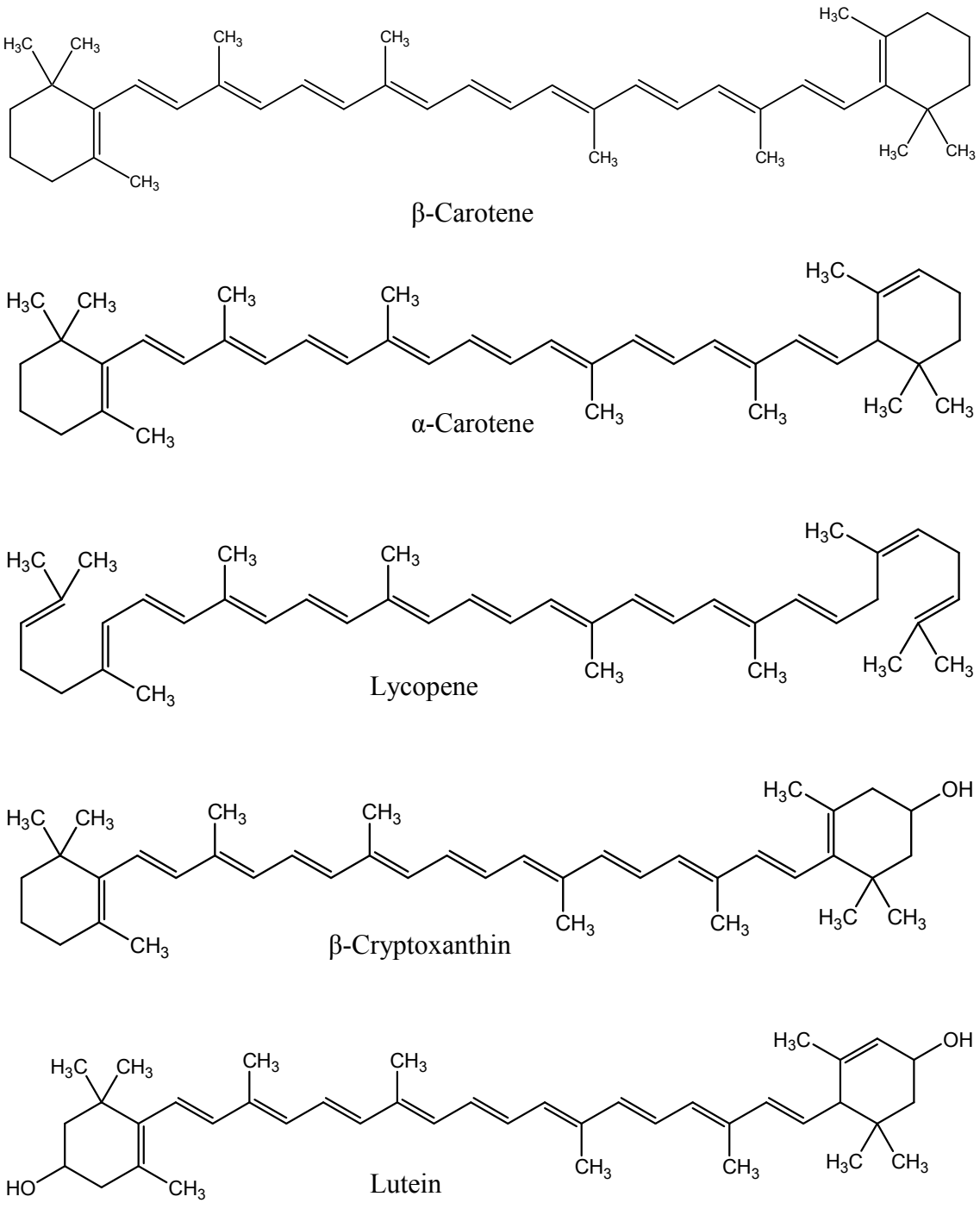


Figure 2.1.7. Structures of some major dietary carotenoids.

In recent years the antioxidant properties of carotenoids have been the major focus of research (Paiva and Russell, 1999). Carotenoid pigments play important functions in

photosynthesis and photoprotection in plant tissues. The photoprotection role of carotenoids originates from their ability to quench and inactivate reactive oxygen species such as singlet oxygen formed from exposure of light and air. This photoprotection role is also associated with its antioxidant activity in human health (Liu, 2004).

Carotenoids can react with free radicals and become radicals themselves. They functions as a chain-breaking antioxidant in a lipid environment, especially under low oxygen partial pressures (Burton and Ingold 1984; Rao and Rao, 2007). The peroxyl radicals ($ROO^{\bullet}$) formed from lipids (especially polyunsaturated phospholipids) are very damaging to cells. The extensive systems of double bonds make carotenoids susceptible to attack by peroxyl radicals, resulting in the formation of inactive products (Chew, 1995).

$$RH + \text{Oxidant initiator} \longrightarrow R^{\bullet}$$

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + \text{Carotenoids} \longrightarrow \text{Formation of inactive products}$$

Their reactivity depends on the length of the chain of conjugated double bonds and the characteristics of the end groups. Carotenoid radicals are stable by virtue of the delocalization of the unpaired electron over the conjugated polyene chain of the molecules. This delocalization also allows addition reactions to occur at many sites on the radical (Britton, 1995). Carotenoids at sufficient concentrations can prevent lipid oxidation and related oxidative stress (Liu, 2004).

2.2. Antioxidant activity

The importance of oxidation in the body and in foodstuffs has been widely recognized. Oxidative metabolism is essential for the survival of cells. Maintenance of equilibrium between free radical production and antioxidant defenses (enzymatic and non enzymatic) is an essential condition for normal organism functioning. When this equilibrium is displaced to the production of free radicals we say that the organism is in oxidative stress. In this situation, excess free radicals may damage cellular lipids, proteins and DNA, inhibiting normal function and leading to various diseases. In aerobic organisms, the free radicals are constantly being produced during the normal cellular functioning, mainly in the form of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). Exposition of the organism to free radicals has led to the development of endogenous defense mechanisms to eliminate them. These defenses were the response of evolution to the inevitability of ROS production in aerobic conditions. Natural products with antioxidant activity may help the endogenous defense system. In this perspective antioxidants present in the diet assume a major importance as possible protector agents reducing oxidative damage (Ferreira and Abreu, 2007). Furthermore, oxidation can also affect foods, where it is one of the major causes of chemical spoilage (Colbert and Decker, 1991), resulting in rancidity and/or deterioration of the nutritional quality, colour, flavour, texture and safety of foods (Shahidi et al., 1992). Defense mechanisms against the effects of excessive oxidations are provided by the action of various antioxidants (Antolovich et al.2002).

2.2.1. Oxidative stress

A free radical is defined as any atom or molecule possessing unpaired electrons in the outer orbit (Halliwell and Gutteridge, 1999). They are generally unstable and very reactive. Oxygen and nitrogen free radicals can be converted to other non-radical reactive species, such as hydrogen peroxide, hypochlorous acid (HClO), hypobromous acid (HBrO), and peroxynitrite ($ONOO^-$). ROS, RNS, and reactive chlorine species are produced in animals and humans under physiologic (normal cellular metabolism of aerobic cells) and pathologic conditions (Aust and Sringen, 1982; Pryor et al., 1982;

Evans and Halliwell, 2001). Reactive radical and non-radical species are summarized in **Table 2.2.1**.

Table 2.2.1. Reactive oxygen species (ROS) and reactive nitrogen species (RNS)*

	Radicals	**Nonradicals**
ROS	Superoxide, $O_2^{\bullet -}$	Hydrogen peroxide, H_2O_2
	Hydroxyl, $OH^{\bullet}$	Hypochlorous acid, HOCl
	Peroxyl, $RO_2^{\bullet}$	Ozone, O_3
	Alkoxyl, $RO^{\bullet}$	Singlet oxygen, $^1\Delta g$
	Hydroperoxyl, $HO_2^{\bullet}$	
RNS	Nitric oxide, $NO^{\bullet}$	Nitrous acid, HNO_2
	Nitrogen dioxide, $NO_2^{\bullet}$	Dinitrogen tetroxide, N_2O_4
		Dinitrogen trioxide, N_2O_3
		Peroxynitrite, $ONOO^-$
		Peroxynitrous acid, ONOOH
		Nitronium cation, NO_2^+
		Alkyl peroxynitrites, ROONO

*(Halliwell, 1996)

ROS and RNS are well recognised for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems (Valko et al., 2006). Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular responses to anoxia, as for example in defense against infectious agents and in the function of a number of cellular signalling systems. One further beneficial example of ROS at low/moderate concentrations is the induction of a mitogenic response (Valko et al., 2007).

Nitric oxide (NO) is one of the most widespread signaling molecules and participates in virtually every cellular and organ function in the body. Physiologic levels of NO produced by endothelial cells are essential for regulating the relaxation and proliferation of vascular smooth muscle cells, leukocyte adhesion, platelet aggregation, angiogenesis, thrombosis, vascular tone, and hemodynamics (Ignarro et al., 1999). In addition, NO produced by neurons serves as a neurotransmitter, and NO generated by activated macrophages is an important mediator of the immune response (Freidovich, 1999).

However, as oxidants and inhibitors of enzymes containing an iron-sulfur center, free radicals and other reactive species cause the oxidation of biomolecules, which leads to cell injury and death (Freidovich, 1999; McCord, 2000; Fang et al., 2002). There is an

increased evidence for the participation of free radicals in the etiology of various diseases like cancer, diabetes, cardiovascular diseases, autoimmune disorders, neurodegenerative diseases, aging, etc. (Bandopadhyay et al., 1999). The harmful effect of free radicals causing potential biological damage is termed oxidative stress and nitrosative stress (Kovacic et al., 2001; Ridnour et al., 2005; Valko et al., 2001). This occurs in biological systems when there is an overproduction of ROS/RNS on one side and a deficiency of biological antioxidants on the other (Valko et al., 2007).

Oxidative stress results from the metabolic reactions that use oxygen and represents a disturbance in the equilibrium status of prooxidant/antioxidant reactions in living organisms. The excess ROS can damage cellular lipids, proteins or DNA inhibiting their normal function. Because of this, oxidative stress has been implicated in a number of human diseases as well as in the ageing process (Valko et al., 2007). For example, radiation-induced ROS markedly alter the physical, chemical and immunologic properties of superoxide dismutase (SOD) (Fang 1991), which further exacerbates oxidative damage in cells. The cytotoxic effect of free radicals is deleterious to mammalian cells and mediates the pathogenesis of many chronic diseases, but it is also responsible for the killing of pathogens by activated macrophages and other phagocytes in the immune system (McCord, 2000; Fang et al., 2002).

The delicate balance between beneficial and harmful effects of free radicals is a very important aspect of living organisms. Cells often tolerate mild oxidative stress by up-regulating synthesis of antioxidant defense systems in an attempt to restore the balance (Halliwell, 1996); this is achieved by mechanisms called “redox regulation”. The process of “redox regulation” protects living organisms from various oxidative stresses and maintains “redox homeostasis” by controlling the redox status in vivo (Dröge, 2002).

Oxidative stress can be formed in living organisms by both endogenous and exogenous sources. Endogenous sources of free radicals include normal aerobic respiration, peroxisomes and stimulation of polymorphonuclear leucocytes and macrophages (Irshad et al., 2002). The exogenous source for the occurrence of oxidative stress may occur in several ways (**Figure 2.2.1**). Malnutrition may lead to inadequate dietary intake of antioxidants such as α-tocopherol, ascorbic acid, sulfur-containing amino acids (needed for reduced glutathione (GSH) synthesis), or riboflavin (needed to make the flavin adenine dinucleotide (FAD) cofactor of glutathione reductase). Lack of dietary protein may lead to inadequate synthesis of metal ion binding proteins. Excess of $O_2^{\bullet-}$

and H_2O_2 is produced, e.g., by exposure to drugs or toxins, ionizing radiation, tobacco smoke, pollutants, pesticides and organic solvents that are metabolized to produce free radicals, or by excessive activation of "natural" radical-producing systems (e.g., phagocytes in chronic inflammatory diseases) (Halliwell and Gutteridge, 1990; Halliwell 1996; Irshad et al., 2002).

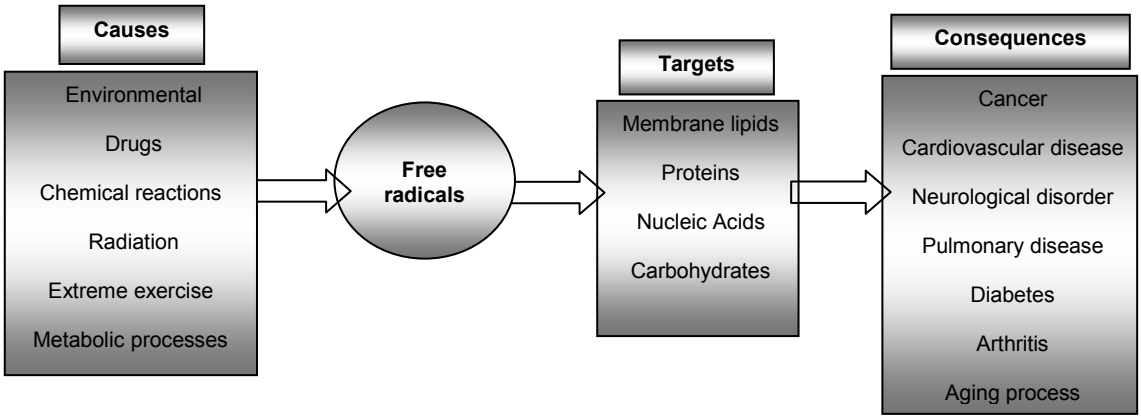


Figure 2.2.1. Free radicals action major causes and consequences (Ferreira and Abreu, 2007).

Free radicals can cause a wide range of toxic oxidative reactions like initiation of the peroxidation of the membrane lipids leading to the accumulation of lipid peroxides, direct inhibition of mitochondrial respiratory chain enzymes, fragmentation or random cross linking of molecules like DNA, enzymes and proteins which ultimately leads to cell death (Halliwell and Gutteridge, 1999).

Damaged tissues undergo more free radical reactions than healthy ones (Halliwell and Gutteridge, 1992). In most human diseases, oxidative stress is a secondary phenomenon, not the primary cause of the disease (Gutteridge, 1993). This does not mean that oxidative stress is unimportant. For example, secondary oxidative damage to lipids in blood vessel walls is a significant contributor to the development of atherosclerosis, and low dietary vitamin E intake is a risk factor. Dietary vitamin E requirement is probably raised if the percentage of polyunsaturated fatty acids in the diet is increased, a phenomenon well-known in animals but not yet fully explored in humans. DNA damage by ROS and RNS probably contributes to the age-related development of cancer (Halliwell, 1996). Oxidative stress contributes to tissue damage in rheumatoid arthritis, inflammatory bowel diseases (Halliwell and Gutteridge, 1989), and Parkinson's disease (Jenner, 1994). Evidence is growing that the major killers, cardiovascular disease and

cancer, can be prevented or delayed to some extent by dietary changes, such as reduction in fat intake and increased consumption of fruits, grains and vegetables (Willett, 1994). Since our endogenous antioxidant defenses are not 100% efficient, it is reasonable to propose that dietary antioxidants are important in diminishing the cumulative effects of oxidative damage over the long human lifespan, and that they account for some of the beneficial effects of fruits, grains and vegetables (Halliwell, 1996).

Thus, there are "two faces" of free radicals in biology in that they serve as signaling and regulatory molecules at physiologic levels, but also as highly deleterious and cytotoxic oxidants at pathologic levels (Freidovich, 1999; Fang et al., 2002).

2.2.1.1. Reactive oxygen species (ROS)

Radicals derived from oxygen represent the most important class of radical species generated in living systems. Molecular oxygen (O_2) has a unique electronic configuration and is itself a radical. The addition of one electron to O_2 forms the superoxide anion radical ($O_2^{\bullet -}$). Superoxide anion, arising either through metabolic processes or following oxygen "activation" by physical irradiation is considered the "primary" ROS. The production of superoxide occurs mostly within the mitochondria of a cell (Cadenas et al., 2000). The mitochondrial electron transport chain is the main source of ATP in the mammalian cell and thus is essential for life. During energy transduction, a small number of electrons "leak" to oxygen prematurely, forming the oxygen free radical superoxide, which has been implicated in the pathophysiology of a variety of diseases. Measurements on submitochondrial particles suggest an upper limit of 1–3% of all electrons in the transport chain "leaking" to generate superoxide instead of contributing to the reduction of oxygen to water (Kovacic et al., 2001; Valko et al., 2001).

Even though the superoxide is not a very active radical, it can interact with other molecules generating what is considered as "secondary" ROS, such as hydrogen peroxide, hydroxyl radical. The production of these radicals takes place? either directly or prevalently through enzyme- or metal-catalysed processes (Valko et al., 2005). Hydroxyl radical ($OH^{\bullet}$) has a very short life time but is considered to be the most toxic among all ROS, being the most responsible radical for the attack of DNA molecules, damaging purins and pyrimidines and the structure of desoxiribose DNA (Ferreira and

Abreu, 2007). Hydroxyl radical is the neutral form of the hydroxide ion and it is formed by an electron transfer from transition metals to H_2O_2. $OH^{\bullet}$ interacts with biomolecules immediately after generation (Pastor et al., 2000; Goetz and Luch, 2008).

In the presence of copper or iron ions, electron donors such as NADPH, catechin, hydroquinone, ascorbic acid or glutathione (GSH) facilitate the formation of $OH^{\bullet}$ from H_2O_2 (referred to as "Fenton reaction"). In particular, low molecular weight complexes of iron contribute to the formation of substantial amounts of $OH^{\bullet}$. Due to its capability to generate ROS, iron may contribute to the peroxidation of cellular macromolecules such as lipids and thus can exert strong cytotoxicity if not tightly controlled via binding to specific iron storage proteins such as ferritin. Certain xenobiotic compounds such as catechols are known to trigger the release of iron from ferritin and thus initiate lipid peroxidation (LPO) in those cells exposed (Valko et al., 2007, Goetz and Luch, 2008). It is assumed that $OH^{\bullet}$ contributes to a plethora of diseases and biological phenomena including cancer, aging, diabetes, phagocytosis, cataractogenesis, ischemia-reperfusion injury, quinone toxicity, and radiation injury (Halliwell and Gutteridge, 1999).

Similar to $O_2^{\bullet-}$ and due to its stability, generation of H_2O_2 in certain compartments may also pose a risk to biomolecules at distant sites. The main sources of cellular H_2O_2 are (i) peroxisome metabolism, (ii) autoxidation of reactive chemicals during redox-cycling at both microsomal and mitochondrial sites of electron-transport, and (iii) SOD activity (Goetz and Luch, 2008). H_2O_2 is mainly removed by thioredoxin-dependent peroxidases (peroxiredoxins), glutathione peroxidases (GSH-Px), and peroxisomal catalase.

Simultaneous knockout of GSH-Px1 and GSH-Px2 in mice promotes the development of intestinal cancers (Chu et al., 2004) and UV-dependent squamous cell carcinomas of the skin (Walshe et al., 2007). However, regular expression and activity of these and other antioxidant enzymes contribute more to the prevention of tumor initiation than to suppression of tumor growth (Neumann and Fang, 2007).

Additional reactive radicals derived from oxygen that can be formed in living systems are peroxyl radicals ($ROO^{\bullet}$). The simplest peroxyl radical is perhydroxyl radical ($HOO^{\bullet}$, $HO_2^{\bullet}$), which is the protonated form of $O_2^{\bullet-}$ and is usually termed either hydroperoxyl radical or perhydroxyl radical. It has been demonstrated that hydroperoxyl radical initiates fatty acid peroxidation by two parallel pathways: fatty acid hydroperoxide (LOOH)-independent and LOOH-dependent (Aikens and Dix, 1991). The LOOH-dependent pathway of $HO_2^{\bullet-}$ initiated fatty acid peroxidation may be relevant to mechanisms of LPO initiation *in vivo* (Valko et al., 2007).

2.2.1.2. Reactive nitrogen species (RNS)

Nitric oxide is a small molecule that contains one unpaired electron on the antibonding 2 π^*_y orbital and is, therefore, a radical. It is generated in biological tissues by specific nitric oxide synthases (NOS), which metabolise arginine to citrulline with the formation of $NO^{\bullet}$ *via* a five electron oxidative reaction (Ghafourifar and Cadenas, 2005). $NO^{\bullet}$ is an abundant reactive radical that acts as an important oxidative biological signalling molecule in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defense mechanisms, smooth muscle relaxation and immune regulation (Bergendi et al., 1999), but too much $NO^{\bullet}$ (like too much $O_2^{\bullet-}$) is toxic (Halliwell, 1996).

$NO^{\bullet}$ has a half-life of only a few seconds in an aqueous environment. $NO^{\bullet}$ has greater stability in an environment with a lower oxygen concentration. However, since it is soluble in both aqueous and lipid media, it readily diffuses through the cytoplasm and plasma membranes and once in the extracellular medium, $NO^{\bullet}$ reacts with oxygen and water to form nitrate and nitrite anions (Chiueh, 1999).

Overproduction of reactive nitrogen species is called nitrosative stress. This may occur when the generation of reactive nitrogen species in a system exceeds its ability to neutralise and eliminate them. Excess $NO^{\bullet}$ production is thought to be an important tissue injury mechanism in such conditions as chronic inflammation, stroke and septic shock (Halliwell and Gutteridge, 1999).

Nitrosative stress may lead to nitrosylation reactions that can alter the structure of proteins and so inhibit their normal function. Cells of the immune system produce both the superoxide anion and nitric oxide during the oxidative burst triggered during inflammatory processes (Valko et al., 2007). Under these conditions, $NO^{\bullet}$ can react with $O_2^{\bullet-}$ to produce significant amounts of a much more oxidatively active molecule, peroxynitrite anion ($ONOO^-$), which is a potent oxidising agent that can cause DNA fragmentation and lipid oxidation (Goetz and Luch, 2008).

Because of the longer half-life of $NO^{\bullet}$ as compared to $O_2^{\bullet-}$, $ONOO^-$ most likely is formed in the same cellular compartment as $O_2^{\bullet-}$ (i.e., mitochondria). On the other hand, cytosolic formation of $ONOO^-$ is amplified when concentrations of L-arginine are limited, since under these conditions NOS catalyze the formation of both $NO^{\bullet}$ and $O_2^{\bullet-}$. Due to its short half-life, subcellular migration of $ONOO^-$ will occur only within short

ranges but may include transmembrane migration through anion channels. $ONOO^-$ can further react with carbon dioxide to give nitroso-peroxocarboxalate that rapidly breaks down into nitrogen dioxide and carbonate radical anions. Alternatively, upon protonation $ONOO^-$ may dissociate into nitrogen dioxide and $OH^\bullet$ (Radi et al., 2001). The main cellular targets of $ONOO^-$ are thiol group containing molecules, transition metals and carbon dioxide (Goetz and Luch, 2008).

2.2.1.3. Processes of lipid oxidation

The nature of cell damage induced by ROS depends on the sites of ROS formation. Since compartmentalization is crucial for cell viability, damage to membrane structures usually triggers initiation of cell death. ROS-mediated impairment of membrane function can either occur directly through oxidation of polyunsaturated fatty acids present in lipids, or indirectly through inhibition of lipid synthesis, fatty acid desaturation, or activation of lipases. Since subcellular membranes contain high amounts of polyunsaturated fatty acids, intermediate formation of carbon-centered radicals within membranes results in peroxidation of many fatty acids depending on the prevailing concentrations of O_2 and transition metals (Goetz and Luch, 2008).

Lipid oxidation is important in food deterioration and oxidative modification of low-density lipoprotein (LDL). Lipid oxidation proceeds (Adegoke et al., 1998) *via* three different pathways: (1) non-enzymatic free radical-mediated chain reaction, (2) non-enzymatic, non-radical photo-oxidation and (3) enzymatic reaction (Antolovich et al., 2002). The essential features of oxidation *via* a free radical-mediated chain reaction can be subdivided into four stages: (i) the generation of reactive radicals such as $HO^\bullet$ and $HO_2^\bullet$ and subsequent extraction of hydrogen from biomolecules (initiation), (ii) radical chain propagation, (iii) branching, and (iv) termination (Goetz and Luch, 2008; Antolovich et al., 2002). The process may be initiated by the action of external agents such as heat, light or ionizing radiation or by chemical initiation involving metal ions or metalloproteins (Kanner et al., 1987).

The lipid peroxidation (**Figure 2.2.2**) usually begins with the extraction of a hydrogen atom from the polyunsaturated lipid (LH) chain, through the action of reactive species such as $HO^\bullet$ or H_2O_2. This generates a highly reactive allyl lipid radical ($L^\bullet$) that can react with oxygen to form a lipid peroxyl radical ($LOO^\bullet$). The peroxyl radicals are the chain carriers of the reaction and if not neutralized by antioxidants defenses they will

futher oxidize the lipid, producing hydroperoxides lipids (LOOH), which in turn break down to a wide range of compounds including alcohols, aldehydes, alkyl formates, ketones and hydrocarbons and radicals including the alkoxyl radical ($LO^{\bullet}$). Hydroperoxides can easily be decomposed to form again $L^{\bullet}$ radical (Ferreira and Abreu, 2007).

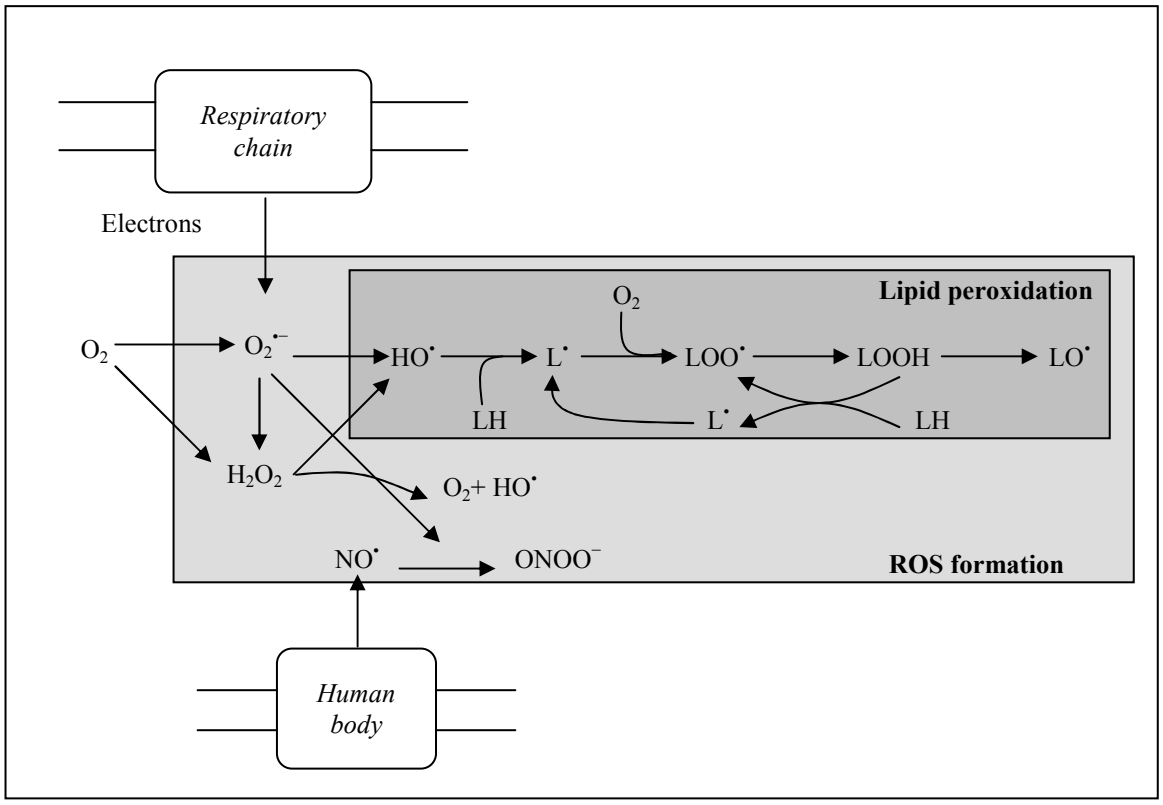


Figure 2.2.2. Summary of some of the major reactions involving reactive oxygen species (ROS) and reactive nitrogen species (RNS). The respiratory chain produces superoxide anion ($O_2^{\bullet-}$) that can be turned into hydrogen peroxide (H_2O_2) and hydroxide radical ($HO^{\bullet}$). These radicals can react with membranes lipids (LH), promoting the process of lipid peroxidation, this will origin the formation of lipid radical ($L^{\bullet}$), peroxy radical ($LOO^{\bullet}$) and lipid hydroperoxides (LOOH). The synthase of nitric oxide in the mitochondrial (NOS) produces nitric oxide ($NO^{\bullet}$), which combines with the superoxide anion to produce peroxynitrile ($ONOO^{-}$). When in excess all these ROS can cause mitochondrial damage and cellular damage (Ferreira and Abreu, 2007).

The breakdown of lipid hydroperoxides often involves transition metal ion catalysis, in reactions analogous to that with hydrogen peroxide, yielding lipid peroxyl and lipid alkoxyl radicals. The termination reactions involve the combination of radicals to form non-radical products (Antolovich et al., 2002).

There are obvious differences between the reactions occurring *in vivo* and in foods (McClements and Decker 2000; Moussata and Akoh, 1997) that may be exposed to elevated temperatures during storage and/or processing. For instance, hydroperoxides decompose readily and spontaneously at 160°C and the peroxy radical concentration

can become relatively high under such conditions, thus leading to the formation of polymers. The measurement of antioxidant activity of certain components *in vivo* requires the definition of the type of free radical formation. At least four different types may be identified as: free iron and the Fenton reaction (Rao and Cederbaum, 1997); mitochondrial lesions and pore reactions leading to apoptosis (Ferrari, 2000); chemically induced free radical formation (Corasaniti et al., 1998), and hydrogen peroxide formation *in vivo* (Baliga et al., 1999).

2.2.2. Antioxidants

Antioxidants can greatly reduce the damage due to oxidants by neutralizing the free radicals before they can attack the cells and prevent damage to lipids, proteins, enzymes, carbohydrates and DNA (Fang et al., 2002). An antioxidant may be defined as "any substance that when present at low concentrations, compared with those of the oxidizable substrate significantly delays or inhibits oxidation of that substrate" (Gutteridge, 1994). Antioxidants represent a class of compounds that vary widely in chemical structure and have varied mechanisms of action. The most important mechanism is their reaction with lipid free radicals, giving inactive products (Pokorný and Korczak, 2001). The mechanisms of antioxidant activity are shown in **Table 2.2.2**.
For convenience, antioxidants have been traditionally divided into two classes, primary or chainbreaking antioxidants, which react directly with lipid radicals and convert them into stable products, and secondary or preventative antioxidants (Decker et al., 2005). Secondary or preventative antioxidants are compounds that retard the rate of oxidation; this may be achieved in a number of ways: by binding metal ions able to catalyze oxidative processes, scavenging oxygen, absorbing UV radiation, inhibiting enzymes or by decomposing hydroperoxides, including removal of substrate or singlet oxygen quenching (Schwarz et al., 2001).

Table 2.2.2. Mechanisms of antioxidant activity*

Antioxidant class	Mechanism of antioxidant activity	Examples of antioxidants
Proper antioxidants	Inactivating lipid free radicals	Phenolic compounds
Hydroperoxide stabilizers	Preventing decomposition of hydroperoxides into free radicals	Phenolic compounds
Synergists	Promoting activity of proper antioxidants	Citric acid, ascorbic acid
Metal chelators	Binding heavy metals into inactive compounds	Phosphoric acid, Maillard reaction compounds, citric acid
Singlet oxygen quenchers	Transforming singlet oxygen into triplet oxygen	Carotenes
Substances reducing hydroperoxide	Reducing hydroperoxides in a non-radical way	Proteins, amino acids

*(Hall, 2001)

Primary antioxidants, AH, act by donating an hydrogen atom, and are consumed during the induction period (Gordon, 2001). When present in trace amounts, they may either delay or inhibit the initiation step by reacting with a lipid radical ($L^{\bullet}$) or inhibit the propagation step by reacting with peroxyl ($LOO^{\bullet}$) or alkoxyl radicals ($LO^{\bullet}$) (Antolovich et al., 2002).

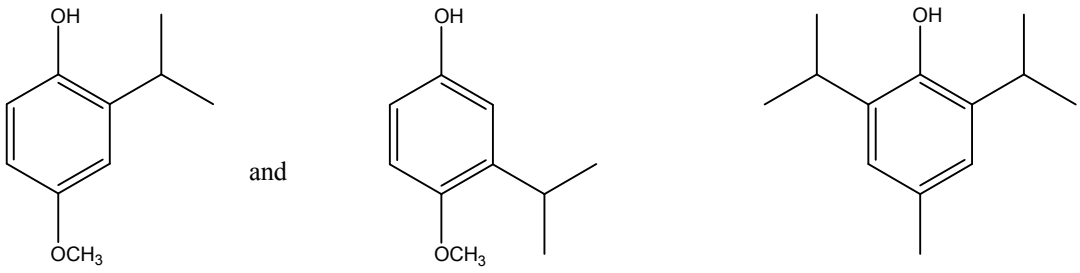
$$L^{\bullet} + AH \longrightarrow LH + A^{\bullet}$$
$$LOO^{\bullet} + AH \longrightarrow LOOH + A^{\bullet}$$
$$LO^{\bullet} + AH \longrightarrow LOH + A^{\bullet}$$

The antioxidant free radical ($A^{\bullet}$) may further interfere with chain propagation reactions by forming peroxy antioxidant compounds.

$$A^{\bullet} + LOO^{\bullet} \longrightarrow LOOA$$
$$A^{\bullet} + LO^{\bullet} \longrightarrow LOA$$

The activation energy of the above reactions increases with increasing A–H and L–H bond dissociation energy. Therefore, the efficiency of the antioxidant increases with decreasing A–H bond strength (Shahidi et al., 1992).

Chain-breaking antioxidants may occur naturally or they may be produced synthetically. A wide range of these antioxidants have been proposed for use in the treatment of various human diseases (Cuzzocrea et al., 2001). There are some synthetic antioxidant compounds such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butylhydroquinone (TBHQ) and propyl gallate (PG). The chemical structures of these compounds are shown in **Figure 2.2.3.** They are added to an extensive variety of foods in order to prevent or retard oxidation, so they are widely used in the food industry (Adegoke et al., 1998) and are included in the human diet (Leclercq et al., 2000; Umamaheswari and Chatterjee 2008).

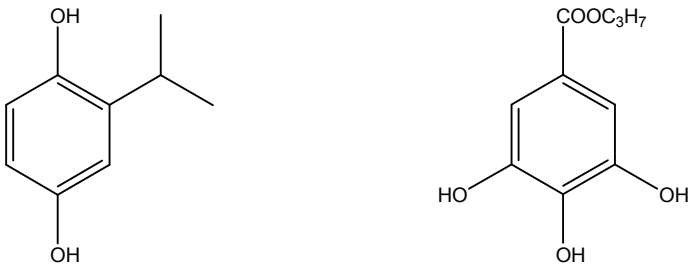


4-methoxy-2-*tert*-butylphenol (2-BHA)

4-methoxy-3-*tert*-butylpheno (3-BHA)

2,6-di-*tert*-butyl-*p*-hydroxytoluene (BHT)

butylated hydroxyanisole (BHA)



tert-butylhydroquinone (TBHQ)

propyl gallate (PG)

Figure 2.2.3. Chemical structures of food-grade synthetic phenolic antioxidants.

The use of naturally occurring antioxidants has been promoted because of concerns regarding the safety of synthetic antioxidants (Antolovich et al., 2002). For example, α-tocopherol (the most active form of vitamin E) is more effective than synthetic racemic

α-tocopherol, primarily because α-tocopherol transfer protein selectively recognizes natural α-tocopherol (Shi et al., 2001). In addition, the possible activity of synthetic antioxidants as promoters of carcinogenesis has become a concern. BHA and related antioxidants have been suggested to have toxic effects like liver damage and mutagenesis (Grice, 1986; Wichi, 1988). Therefore, replacing synthetic antioxidants with natural alternatives, or simply replacing all synthetic food additives with natural choices, has attracted great interest over the past two decades (Wanasundara and Shahidi, 1998).

2.2.2.1. Natural defense against oxidative stress and antioxidants

Exposure to free radicals from a variety of sources has led organisms to develop a series of defense mechanisms (Cadenas, 2000). Defense mechanisms against free radical-induced oxidative stress involve: (i) preventive mechanisms, (ii) repair mechanisms, (iii) physical defenses, and (iv) antioxidant defenses (Valko et al., 2007). The removal of free radicals is achieved through two major classes of antioxidants that can be classified into enzymatic and non-enzymatic. The enzymatic antioxidants are produced endogenously and include superoxide dismutase (SOD), glutathione peroxidases (GSH-Px), catalase (CAT), glutathione redutase (GPH-R) among others. The non-enzymatic antioxidants obtained from natural sources are represented by ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), carotenoids, flavonoids, tannins and other antioxidants (Valko et al., 2007; Fang et al., 2002; Lee et al., 2004). The management of oxidative stress by GSH and other antioxidants is shown in **Figure 2.2.4**. Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants, this balance is essential for the survival of organisms and their health.

Superoxide dismutase immediately converts $O_2^{\bullet -}$ to H_2O_2, which is then detoxified to water either by CAT in the peroxysomes or by GSH-Px in the mitochondria, cytosol or nucleus. Another enzyme that is important is GSH-R, which regenerates GSH that is used as a hydrogen donor by GSH-Px during the elimination of H_2O_2 or other peroxydes (Johansen et al., 2005). GSH-Px can also transform hydroperoxide lipids into alcohols (LOH) (Ferreira and Abreu, 2007).

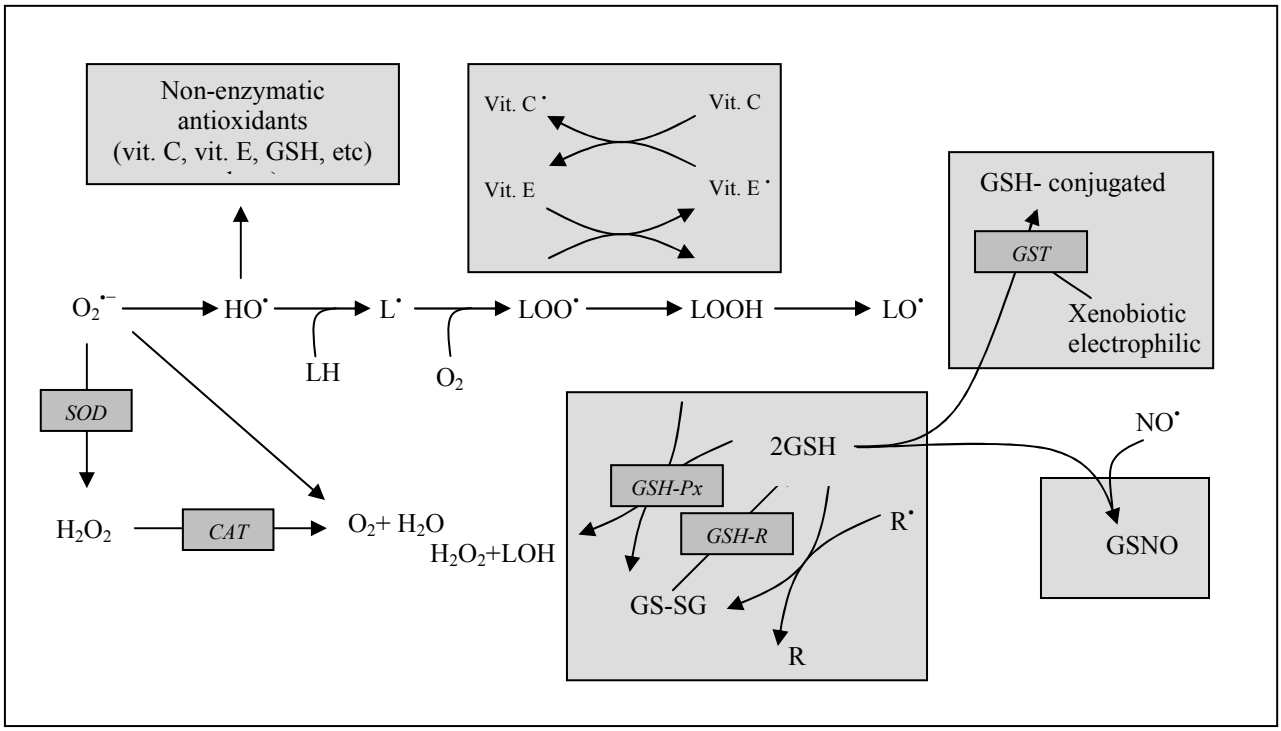


Figure 2.2.4. Major endogenous antioxidant defenses of the cell. The ROS and the RNS are represented in a similar way as in **figure 2.2.2**. The antioxidant defenses are represented in rectangles and enzymes represented in italics. ROS: superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), membrane lipids (LH), lipid radical ($L^{\bullet}$), peroxyl radical ($LOO^{\bullet}$), lipid hydroperoxides (LOOH), nitric oxide ($NO^{\bullet}$), alcohols (LOH). Antioxidant defenses: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione redutase (GPH-R), glutathione (GSH), glutathione disulphide (GS-SG), α-tocopherol or vitamin E (vit. E), vitamin E radical (vit. $E^{\bullet}$), vitamin C (vit. C), vitamin radical (vit. $C^{\bullet}$), non-radical (R), radical ($R^{\bullet}$), S-nitrosoglutathione (GSNO), glutathione-S-transferases (GST) (Ferreira and Abreu, 2007).

Glutathione (L-g-glutamyl-L-cysteinylglycine) is the most abundant non proteic thiol-containing substance of low molecular weight in cells and is the principal thiol involved in the antioxidant cellular defence (Fang et al., 2002). It is a tripeptide composed of glutamic acid, cysteine, and glycine, and its active group is represented by the thiol (–SH) of cysteine residue. Glutathione is a ubiquitous molecule that is produced in all organs, especially in the liver (Pastore et al., 2003).

As a major component of the cellular antioxidant system, GSH has the following characteristics: 1) GSH in the diet can be partly absorbed from the small intestine and can be synthesized again, so that GSH is an exogenous and endogenous antioxidant; 2) in the neutralization process of ROS, GSH is oxidized to glutathione radical ($GS^{\bullet}$) being a pro-oxidant radical, $GS^{\bullet}$ can react with another $GS^{\bullet}$ to yield glutathione disulphide (GS-SG), which can be further reduced to GSH by the GSH-R; 3) GSH can react with a variety of xenobiotic electrophilic compounds in the catalytic reaction of glutathione-S-transferases (GST), making these compounds soluble and more easily transferable; 4) GSH effectively scavenges ROS ($HO^{\bullet}$, H_2O_2, $LOO^{\bullet}$ and $ONOO^{-}$) directly and indirectly

as a cofactor of several detoxifying enzymes, e.g. GSH-Px, GST and others; 5) GSH can conjugate with $NO^{\bullet}$, resulting in the formation of a S-nitrosoglutathione adduct (GSNO), which is cleaved by the thioredoxin system to release GSH and NO; 6) GSH interacts with glutaredoxin and thioredoxin (thiol-proteins), which play important roles in the regulation of cellular redox homeostasis, and DNA synthesis, and 7) GSH participates in amino acid transport through the plasma membrane (Fang et al., 2002; Masella et al., 2005; Valko et al., 2007).

The capacity of glutathione to regenerate the most important antioxidants is linked with the redox state of the glutathione disulphide-glutathione couple (GSSG/2GSH) (Pastore, et al., 2003). GSH is also able to regenerate the most important antioxidants, vitamins C and E, back to their active forms; GSH can reduce the tocopherol radical of vitamin E directly, or indirectly, *via* reduction of semidehydroascorbate to ascorbate (Valko et al., 2007). Vitamin E can transfer its phenolic hydrogen to a peroxyl free radical of a peroxidized PUFA, thereby breaking the radical chain reaction and preventing the peroxidation of PUFA in cellular and subcellular membrane phospholipids. As a reducing agent, vitamin C reacts with a vitamin E radical to yield a vitamin C radical while regenerating vitamin E. Like a vitamin E radical, a vitamin C radical is not a reactive species because its unpaired electron is energetically stable (Fang et al., 2002).

2.2.3. Antioxidant activity assays

Antioxidant activity cannot be measured directly but rather by the effects of the antioxidant in controlling the extent of oxidation (Antolovich et al., 2002).

Numerous tests have been developed for measuring the antioxidant capacity of food and biological samples. However, there is no universal method that can measure the antioxidant capacity of all samples accurately and quantitatively. Clearly, matching radical source and system characteristics to antioxidant reaction mechanisms is critical in the selection of appropriate assessing antioxidant capacity (AOC) assay methods, as is consideration of the end use of the results (Prior et al., 2005; Wrigth et al., 2001).

A standardized method for AOC should meet the following "ideal" requirements: measure chemistry actually occurring in potential application(s); utilize a biologically relevant radical source; simple; use a method with a defined endpoint and chemical mechanism; instrumentation readily available; good within-run and between-day reproducibility; adaptable for assay of both hydrophilic and lipophilic antioxidants and

use of different radical sources; adaptable to "high-throughput" analysis for routine quality control analyses (Prior et al., 2005).

Due to the chemical diversity of antioxidant compounds present in foods, it is unrealistic to separate each antioxidant component and study it individually. In addition, levels of single antioxidants in food do not necessarily reflect their total antioxidant capacity because of the possible synergistic interactions among the antioxidant compounds in a food mixture. Methods of assessing antioxidant capacity fall into two broad categories reflecting the focus on radicals scavenging activity/lipid inhibition *in vitro* and bioactivity *in vivo* (Magalhães et al., 2008).

2.2.3.1. Radical scavenging capacity

Currently, the most widely used methods for measuring antioxidant activity are those that involve the generation of radical species. These approaches have been applied to the estimation of antioxidant activity in aqueous systems, but not as much for lipid-soluble antioxidants in nonpolar system (Fukumoto and Mazza, 2000; Huang et al., 2002; Awike et al., 2003; Zaporozhets et al., 2004). Based on the chemical reactions involved, the major antioxidant capacity assays can be roughly divided into two categories: (1) hydrogen atom transfer (HAT) reaction based assays, and (2) single electron transfer (ET) reaction based assays. These reactions may occur in parallel and usually occur together in all samples (Prior et al., 2005). HAT and ET measure the radical (or oxidant) scavenging capacity of a sample instead of the preventive antioxidant capacity (Prior et al., 2005).

The factors that determine the mechanism of antioxidant activity in radical deactivation in a given system are antioxidant structure and properties, antioxidant solubility and partition coefficient, pH and nature of the system (Huang et al., 2005). The mechanism and the efficacy of antioxidants may be also determined by bond dissociation energy and ionization potential (Wright et al., 2001).

The majority of HAT-based assays are kinetics based, and involve a competitive reaction scheme, in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo compounds. This type of assays detects the capacity of an antioxidant to quench free radicals by donating hydrogen (AH = any H donor) (Prior et al., 2005). Therefore, a synthetic free radical generator, an

oxidized molecular probe and an antioxidant are the main components of any HAT-based method (Wright et al., 2001).

$$X^{\bullet} + AH \longrightarrow XH + A^{\bullet}$$

HAT-based assays are not dependent on the nature of the solvent or pH (Wright et al., 2001). They are usually relatively rapid, typically completed in seconds to minutes. Reducing agents, including metals, interfere with HAT-based reactions and may lead to erroneously high apparent capacities (Prior et al., 2005).

Single electron transfer based methods measure the capability of a potential antioxidant to transfer one electron to reduce an oxidant, including metals, carbonyls and radicals which changes color when reduced (Wright et al., 2001). The degree of colour change is correlated with the sample's antioxidant concentrations.

$$X^{\bullet} + AH \longrightarrow X^{-} + AH^{\bullet+}$$

$$AH^{\bullet+} + H_2O \longleftrightarrow A^{\bullet} + H_3O^{+}$$

$$X^{-} + H_3O^{+} \longrightarrow XH + H_2O$$

$$M^{3+} + AH \longrightarrow AH^{+} + M^{2+}$$

Single electron transfer based reactions are pH dependent. Antioxidant capacity calculations in these reactions are based on the percent decrease in product rather than kinetics, since ET-based reactions are usually slow and can require long times to reach completion (Wright et al., 2001). The presence of trace components and contaminants (particularly metals) in samples could account for the high variability and poor reproducibility and consistency of results (Sartor et al., 1999).

In the present study, the following assays were used to assess the antioxidant capacities of samples: radical-scavenging activity (RSA) assay (HAT), reducing power assay (ET) and β-carotene-linoleic acid (linoleate) assay (HAT).

2.2.3.1.1. Diphenylpicrylhydrazyl radical ($DPPH^{\bullet}$) scavenging activity

This method was given by Brand-Williams et al. (1995) and later modified by Sánchez-Moreno et al. (1998) and is one of the most extensively used antioxidant assay.

The 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$, **Figure 2.2.5**) is a stable organic nitrogen radical, is commercially available and has a deep purple colour. The RSA assay measures the reducing capacity of antioxidants toward DPPH$^{\bullet}$. Upon reduction, the colour of DPPH$^{\bullet}$ solution fades and this colour change is conveniently monitored spectrophotometrically at 517 nm. Therefore, test compounds with high antioxidant activity result in a rapid decline in the absorbance of the DPPH$^{\bullet}$ (Amarowicz et al., 2004; Antolovich et al., 2002).

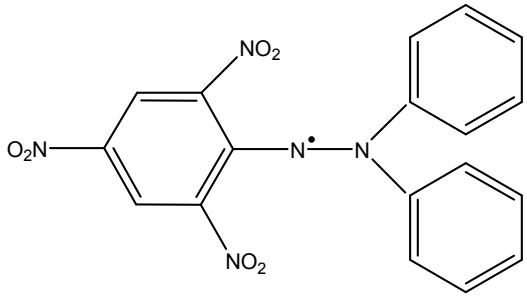


Figure 2.2.5. Chemical structure of 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$).

When a solution of DPPH$^{\bullet}$ is mixed with a substance that can donate a hydrogen atom, the reduced form of the radical is generated accompanied by loss of colour (Ali et al., 2008). Representing the DPPH$^{\bullet}$ by X$^{\bullet}$ and the donor molecule by AH, the primary reaction is:

$$X^{\bullet} + AH \longrightarrow XH + A^{\bullet}$$

DPPH$^{\bullet}$ remains unaffected by certain side reactions (*e.g.*, metal-ion chelation and enzyme inhibition), which is dissimilar to laboratory generated free radicals such as the hydroxyl radical and the superoxide anion (Amarowicz et al., 2004).

The interpretation of results is complicated when the test compounds have spectra that overlap DPPH$^{\bullet}$ at 517 nm, *e.g.*, carotenoids (Nomura et al., 1997). Either radical reaction or reduction can decolorize DPPH$^{\bullet}$. Small molecules that have better access to the radical site show higher antioxidant capacity with this test. Since DPPH$^{\bullet}$ is a stable nitrogen radical, unlike the highly reactive and transient peroxyl radicals involved in lipid peroxidation, many antioxidants that react quickly with peroxyl radicals may react slowly with, or may even be inert to DPPH$^{\bullet}$, due to steric inaccessibility (Prior et al., 2005). It was reported that the reaction of DPPH$^{\bullet}$ with eugenol was reversible (Bondet

et al., 1997), which leads to falsely low readings for antioxidant capacity of samples containing eugenol and other phenols with similar structures, *e.g.*, *o*-methoxyphenol.

2.2.3.1.2. Reducing power

The transformation of Fe^{3+} into Fe^{2+} in the presence of various fractions is measured to determine the reducing power ability. The antioxidant present cause the reduction of Fe^{3+}/ferricyanide complex ($FeCl_3/K_3Fe(CN)_6$) to the ferrous form (Fe^{2+}) (Chung et al., 2002). Therefore, depending on the reducing power of the test compounds, the yellow colour of the test solution changes to various shades of green or blue; this can be measured spectrophotometrically at 700 nm (Yen and Chen, 1995; Amarowicz et al., 2004).

The chemistry of iron-based assays may be summarized with the following reaction equation:

$$Fe^{3+}\text{–L} + \text{antioxidant} \longrightarrow Fe^{2+}\text{–L} + \text{oxidized antioxidant}$$

where L is the ferrous-selective chromogenic ligand producing the colored species Fe^{2+} –L as a result of the concerned redox reaction. Since each mole of *n* electron-reductants would produce *n* moles of Fe^{2+} –L, the molar absorptivity of such an antioxidant in the selected iron-based method would be expected to be *n*-times that of Fe^{2+} –L. Either the oxidant species is Fe^{3+} –L or $Fe(CN)_6^{3-}$ (in the composite ferricyanide reagent), the reduction product with the antioxidant, either Fe^{2+} –L or $Fe(CN)_6^{4-}$, respectively, combines with the other reagent component to produce Prussian blue, $KFe[Fe(CN)_6]$, as the colored product. In other words, when Fe^{3+} is used along with $Fe(CN)_6^{3-}$ as the oxidizing agent (in the modified ferricyanide assay), either one of the two reaction pairs occur, which end up with the same product (Prussian blue) (Berker et al., 2007):

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

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

or

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

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

The reducing power assay is considered to be a sensitive method for the "semiquantitative" determination of dilute concentrations of polyphenolics, which participate in the redox reaction (Amarowicz et al., 2004).

2.2.3.1.3. β-carotene bleaching inhibition

Heat-induced oxidation of an aqueous emulsion system of β-carotene and linoleic acid was reported as an antioxidant test reaction by Marco (1968) and then modified by Miller (1971). Decolorization of β-carotene can be monitored by spectrophotometry at 470 nm (Burda and Oleszek 2001); this decolorization can be employed as an essay of antioxidant activity. The β-carotene undergoes a rapid discoloration in the absence of an antioxidant since the free linoleic acid radical attacks the β-carotene molecule, which loses the double bonds and, consequently, loses its characteristic orange color. β-carotene is extremely sensitive to free radical mediated oxidation of linoleic acid (Gutierrez et al., 2006).
Classical antioxidants can donate hydrogen atoms to quench radicals and prevent decolorization of carotenoids:

$$\beta\text{-carotene} - H\ (\text{orange}) + ROO^{\bullet} \longrightarrow \beta\text{-carotene}^{\bullet}\ (\text{bleached}) + ROOH$$

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

An advantage of the β-carotene bleaching method is that it requires no specialized instrumentation. Phenolic antioxidants can neutralize any free radicals formed within the system (*e.g.*, the linoleate free radical) and, consequently, may delay decolorization of β-carotene (Amarowicz et al., 2004).

2.2.3.2. Inhibition of lipid peroxidation

Lipid peroxidation is a complex process and occurs in multiple stages. It is well accepted that antioxidants retard lipid peroxidation in foods and biological samples. Hence, many techniques are available for measuring the oxidation rate of membranes, food lipids, lipoproteins, and fatty acids, which are particularly useful for antioxidant evaluation. However, each technique measures something different, and no method can

be said to be the gold standard for lipid peroxidation measurement. The effect of an antioxidant on lipid peroxidation can be measured mainly by three subcategories: (1) loss of substrates, (2) peroxide assays, and (3) determination of end products. Lipid substrates that can be used include emulsions of, or liposomes made from, fatty acids or fatty acid esters. Oils and melted fats, ground meat, or other food homogenates can also be used. Biological systems can include erythrocytes, isolated lipoproteins (most often low-density lipoproteins or high-density lipoproteins). The other substrate for peroxidation is oxygen, hence, measurement of the rate of oxygen uptake is another overall index of peroxidation. This technique is useful when spectrophotometric interference occurs or toxic chemicals interfere with enzymatic assays (Antolovich et al., 2002).

Peroxide assays included total peroxide value measurements and separation of peroxide intermediates.

The thiobarbituric acid (TBA) test is one of the oldest and most frequently used tests for lipid peroxidation, another test used is the inibition of erythrocyte hemolysis mediated by peroxyl free radicals. Both assays were employed in the present study.

2.2.3.2.1. Thiobarbituric acid reactive substances (TBARS) assay

The TBARS assay was proposed over 40 years ago and is now the most commonly used method to detect lipid oxidation (Kishida et al., 1993). This procedure measures the malondialdehyde (MDA) formed as the split product of an endoperoxide of unsaturated fatty acids resulting from oxidation of a lipid substrate. It is postulated that the formation of MDA from fatty acids with less than three double bonds (*e.g.*, linoleic acid) occurs *via* the secondary oxidation of primary carbonyl compounds (*e.g.*, non-2-enal) (Fernández et al., 1997). The MDA is reacted with thiobarbituric acid (TBA) to form a pink pigment (TBARS, **Figure 2.2.6**) that is measured spectrophotometrically at 532 nm (Ng et al., 2000).

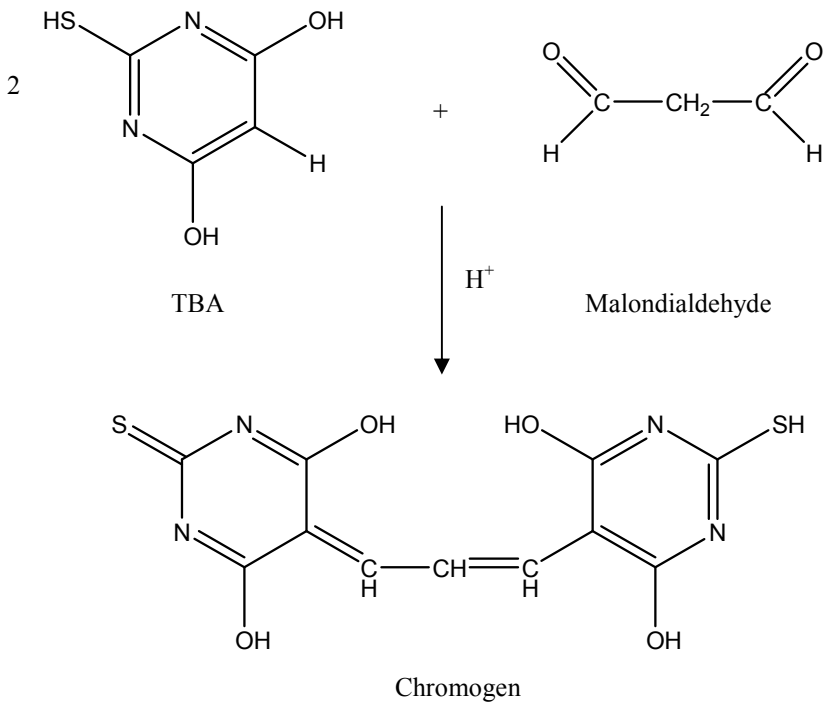


Figure 2.2.6. Chromophore formed by condensation of MDA with TBA.

The procedure involves two distinct steps: the substrate is oxidized with the addition of a transition metal ion such as copper or iron or a free radical source such as 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH, **Figure 2.2.7**) and then the extent of oxidation is determined by addition of TBA and spectrophotometric measurement of the product. Oxidation is inhibited by the addition of an antioxidant and therefore a reduction in the absorbance is seen. Results are typically quantified in terms of percentage inhibition of the oxidation (Antolovich et al., 2002).

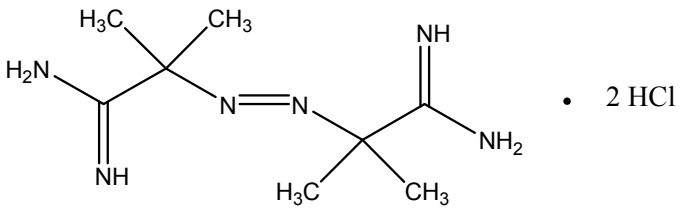


Figure 2.2.7. 2,2'-azobis (2-amidinopropane) dihydrochloride structure.

2.2.3.2.2. Inhibition of erythrocyte hemolysis mediated by peroxyl free radicals

Numerous investigations have used erythrocytes as model systems for studying biomembrane oxidative damage. AAPH (**Figure 2.2.7**) is a free radical initiator that has been used to generate free radicals in the aqueous phase that can attack the erythrocyte membrane and propagate lipid peroxidation, leading to hemolysis (Niki et al., 1988; Mabile et al., 2001). The inhibition of erythrocyte hemolysis can be monitored by spectrophotometry at 540 nm (Ng et al., 2000). Erythrocytes are vulnerable to lipid peroxidation due to their high content of polyunsaturated lipids, their rich oxygen supply, and the presence of transition metals (Zhu et al., 2002). The advantages of this method are that the AAPH decomposes thermally to generate radicals without biotransformation or enzymes and the rate of radical generation is easily controlled by adjusting the concentration of initiator (Niki et al., 1988).

2.3. Antimicrobial activity

Infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat to public health, despite the tremendous progress in human medicine.

Antimicrobial activity of natural extracts and pure compounds can be detected by observing the growth response of various microorganisms to samples that are placed in contact with them. Several methods for detecting antimicrobial activity are available, but since they are not equally sensitive or not based upon the same principle, results will be profoundly influenced by the method (Cos et al., 2006).

The antibacterial and antifungal test methods are classified into three main groups: diffusion, dilution and bioautographic methods. A fourth and upcoming test method is the conductimetric assay, detecting microbial growth as a change in the electrical conductivity or impedance in the growth medium (Sawai et al., 2002). Therein, we will discuss the Agar-diffussion method that has been applied in the present work.

2.3.1. Agar-diffusion method

A very simple way of determining the susceptibility of a microorganism to an antimicrobial agent is to use a microbe-seeded agar plate and to allow the agent to diffuse into the agar medium, which is known as the Kirby-Bauer technique (Mitchell and Carter, 2000).

In the diffusion technique, a reservoir containing the test compound or extract, at a known concentration, is brought into contact with an inoculated medium and the diameter of the clear zone around the reservoir (inhibition diameter) is measured at the end of the incubation period (Cos et al., 2006). The concentration decreases as a function of the square of the distance of diffusion. At some particular distance from the reservoir, the antimicrobial agent is diluted to a point that it no longer inhibits microbial growth. The effectiveness of a particular antimicrobial agent results in the production of growth-inhibition zones that appear as clear areas surrounding the disk from which the agent diffused. The diameter of the zones can be measured with a ruler and the results of such an experiment constitute an antibiogram (Atlas et al., 1995). Agar-diffusion method gives rise to inhibition zones whose diameters correlate with the Minimum Inhibitory Concentrations (MIC) for the microorganisms. MIC means the lowest

concentration, expressed in mg/L, that, under defined in vitro conditions, prevents the growth of microorganism within a defined period of time (Anon, 2000a).

Different types of reservoirs can be used, such as filter paper discs, stainless steel cylinders placed on the surface and holes punched in the medium. The hole-punch method is the only suitable diffusion technique for aqueous extracts, because interference by particulate matter is much less than with other types of reservoirs. To ensure that the sample does not leak under the agar layer, fixed agar is left on the bottom of the hole (Cole, 1994). The small sample requirements and the possibility to test up to six extracts per plate against a single microorganism are specific advantages (Hadacek and Greger, 2000).

The agar diffusion method provides a rapid assessment of antimicrobial activity for any water soluble compound, it is not appropriate for testing non-polar samples or samples that do not easily diffuse into agar (Mitchell and Carter, 2000; Cos et al., 2006). In general, the relative antimicrobial potency of different samples may not always be compared, mainly because of differences in physical properties, such as solubility, volatility and diffusion characteristics in agar (Cos et al., 2006). It is often used commercially to supply basic antimicrobial data during the manufacture, as well as, quality control assurance of the finished product (Ascenzi, 1996; Block, 1991; Paulson, 1999). Antiseptics and disinfectants used in sanitation for food processing and the medical/surgical arena, antibiotics and pesticides for clinical and agricultural use, and preservatives for food, metals, cosmetics, medicines and paints are all examples of compounds that can be tested by this procedure.

2.3.2. Antimicrobial screening

The choice of test organisms depends on the specific purpose of the investigation. In a primary screening, drug-sensitive reference strains are preferably used and should represent common pathogenic species of different classes (Chand et al., 1994).

Various combinations are possible, but the panel should at least consist of a Gram-positive and a Gram-negative bacteria. It has been well-established that Gram-positive bacteria are much more sensitive to drug action than Gram-negative bacteria, which is reflected by a higher number of random ‘hits’ during a screening campaign (Buckner et al., 1996). Extracts with prominent activity against Gram-positive cocci should preferentially also be tested against methicillin-resistant *Staphylococcus aureus* and

vancomycin-resistant *Enterococci*, as they represent the greatest current medical need. The Gram-negative bacteria *Escherichia coli* can be used to test efficacy of an antimicrobial agent. A small set of reference fungi is used for primary screening and includes *Trichophyton mentagrophytes* and *Epidermophyton floccosum* as representatives of the dermatophytes, filamentous fungi can also be screened, such as *Aspergillus niger* and *Fusarium solani* (Cos et al., 2006).

2.3.2.1. Growth medium and inoculums

Although several susceptibility testing media are available in Europe, a clear choice for a reference medium remains to be determined. Mueller-Hinton (MH) agar or broth and tryptic soy agar or broth (TSA or TSB) are general growth media for bacteria, while Sabouraud (SAB) agar or broth is used for fungi. Slight differences in the composition of the growth medium can greatly affect the antibacterial activity of a compound. For example, addition of sheep blood to Mueller-Hinton medium increases the MIC of flavomycin from 0.12 to 256 mg/L (Butaye et al., 2000). Consequently, a definite choice of growth medium is essential to compare different antibacterial compounds or extracts. Mueller-Hinton medium allows good growth of most non-fastidious bacteria and is generally low in antagonists. It also meets the requirements of the NCCLS (USA National Committee for Clinical Laboratory Standards) standard and is recommended as reference medium for agar- and broth-dilution tests (Anon., 2000b; Anon., 2003).

The level of infection, i.e. inoculum concentration can have a profound influence on the antibacterial and antifungal potency of a sample, endorsing the need for standardization of inoculates. A too low inoculum size (e.g., 102 CFU/mL) will create many false-positives, while a too high inoculum size (e.g., 107 CFU/mL) will hamper endpoint reading and increase the chances for false-negatives. Bacterial or yeast inoculates can be prepared from overnight cultures or from existing biofreeze stocks. It is recommended to collect from cultures during the logarithmic growth phase and always to take four or five colonies of a pure culture on agar to avoid selecting an atypical variant (Anon., 2003).

2.3.2.2. Panel of test organisms

Almost all bacteria can be classified as Gram-positive or Gram-negative. The classification relies on the positive or negative results from Gram staining protocol, which uses complex purple dye and iodine. Gram-positive bacteria differ from Gram-negative bacteria in the structure of their cell walls. Both Gram-positive and Gram-negative bacteria have a cell wall made up of peptidoglycan and a phospholipid bilayer with membrane-spanning proteins. The cell walls of Gram-positive bacteria are made up of twenty times as much murein or peptidoglycan than Gram-negative bacteria. These complex polymers of sugars and amino acids cross-link and layer the cell wall. The thick outer matrix of peptidoglycan, teichoic acid, polysaccharides, and other proteins serve a number of purposes, including membrane transport regulation, cell expansion and shape formation. Gram-positive bacteria have more layers of peptidoglycan in their cell walls than Gram-negative, being the reason why they can retain the crystal violet dye and iodine (Singleton, 1999).

Gram-negative bacteria cell walls have a unique outer membrane, a thinner layer of peptidoglycan, and a periplasmic space between the cell wall and the membrane. In the outer membrane, Gram-negative bacteria have lipopolysaccharides (LPS), porin channels and murein lipoprotein, all of which Gram-positive bacteria lack. As opposed to Gram-positive, Gram-negative cells are resistant to lysozyme and penicillin attack. The Gram-negative outer membrane, which contains LPS, an endotoxin, blocks antibiotics, dyes and detergents protecting the sensitive inner membrane and cell wall. LPS are significant in membrane transport of Gram-negative bacteria. LPS, which include O-antigen, a core polysaccharide and a Lipid A, coat the cell surface and works to exclude large hydrophobic compounds, such as bile salts and antibiotics, from invading the cell. O-antigen are long hydrophilic carbohydrate chains (up to 50 sugars long) that extend out from the outer membrane while Lipid A (and fatty acids) anchors the LPS to the outer membrane (Pelczar et al., 1993; Singleton, 1999).

Fungal infections can affect a range of areas in humans from the very surface of the skin to vital systems in the body. Unlike Gram-positive and Gram-negative bacteria, fungi have a bilayered cell membrane with ergosterols. Fungal antibiotics are not designed to target peptidoglycan synthesis because fungi have no peptidoglycan, but they do target the ergosterol, which is unique to fungi. In addition, fungi are eukaryotes as opposed to bacteria that are prokaryotes (Pelczar et al., 1993).

In the present study, the following bacteria and fungi were used to assess the antimicrobial activity of the extracts: Gram-positive bacteria, *Bacillus cereus*, *Bacillus subtilis* and *Streptococcus aureus*, Gram-negative bacteria, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, and fungi, *Candida albicans* and *Cryptococcus neoformas*.

CHAPTER III

RESULTS

3.1. Chemical composition of Portuguese wild mushrooms

3.1.1. Fatty acid and sugar compositions, and nutritional value of five wild edible mushrooms from Northeast Portugal

3.1.1.1. Introduction

More than 2,000 species of mushrooms exist in nature; however, less than 25 species are widely accepted as food and only a few have attained the level of an item of commerce (Smith, 1972; Lindequist et al., 2005). Wild mushrooms are becoming more and more important in our diet for their nutritional (Crisan and Sands, 1978; Breene, 1990; Manzi et al., 1999), organoleptic (Maga, 1981) and pharmacological (Bobek et al., 1991; Bobek et al., 1995; Bobek and Galbavy, 1999) characteristics. The consumption of wild edible mushrooms is increasing due to a good content of proteins and trace minerals (Ogundana and Fagade, 1982; Senatore, 1990; Thimmel and Kluthe, 1998). Some investigations have even contended that the amino acid compositions of mushrooms are comparable to animal proteins (Fink and Hoppenhaus, 1958; Gruen and Wong, 1982). Although, the nutritional potential or implications of the replacement of meat with mushroom requires careful examination which involves detailed chemical and biological studies.

There are no reports about the nutritional value and chemical composition of Portuguese mushrooms. Several studies have been carried out on the chemical composition and nutrition quality of edible mushrooms from different countries, particularly on Spanish (Díez and Alvarez, 2001), Italian (Manzi et al., 2001; Manzi et al., 2004), Turkish (Yildiz et al., 1998), Indian (Longvah and Deosthale, 1998; Agahar-Murugkar and Subbulakshmi, 2005) and Nigerian (Aletor, 1995; Fasidi, 1996) species. The Northeast of Portugal due to climatic conditions and flora diversity is one of the European regions with higher wild edible mushrooms diversity, some of them with great gastronomic relevance. In spite of the immense popularity of this food in the region and their increase exportation to foreign countries (particularly Spain, France and Italy), data

regarding the nutritive value of the wild mushroom varieties available in the region are very meagre.

Herein, we report the chemical composition of five wild edible mushrooms (*Agaricus arvensis, Lactarius deliciosus*, *Leucopaxillus giganteus*, *Sarcodon imbricatus* and *Tricholoma portentosum*), with reference to the contents of dry matter, proteins, fat, carbohydrate and ash. Among the individual components, fatty acid and sugar profiles were obtained by GLC/FID and HPLC/RI, respectively. On the basis of the samples composition, an estimation of the mushrooms nutritional role was also performed.

3.1.1.2. Materials and methods

Samples

Samples of *Agaricus arvensis* (Schaeff.:Fr.) and *Leucopaxillus giganteus* (Sowerby) Singer were colleted under grassland whereas *Lactarius deliciosus* (L.) Gray, *Sarcodon imbricatu*s (L.) P. Karst., and *Tricholoma portentosum* (Fr.) Quél. were collected under live pine trees (*Pinus* sp.), in Bragança (Northeast of Portugal), in autumn 2005. After collection, the mushrooms were grouped by taxon, air-dried in a liophylizator (Ly-8-FM-ULE, Snijders) and powdered before analysis. Taxonomic identification was made according to several authors (Bon, 1988; Courtecuisse, 1999; Courtecuisse and Duhem, 1995; Marchand, 1971; Moser, 1983) and representative voucher specimens were deposited at the herbarium of *Escola Superior Agrária* of *Instituto Politécnico de Bragança*. For all the mushroom species three samples were analysed.

Chemical composition

Samples of mushrooms were analysed for chemical composition (moisture, protein, fat, carbohydrates and ash) using the AOAC procedures (1995). The crude protein content (N × 4.38) of the samples was estimated by the macroKjeldahl method according to Léon-Guzmán et al. (1997); the crude fat was determined by extracting a known weight of powdered mushroom sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600±15 ºC. Total carbohydrates were calculated by difference. Total energy was calculated according to the following equations (Manzi et al., 2004):

Energy (kcal) = 4 × (g protein +g carbohydrate) + 9 × (g lipid);

Energy (kJ) =17 × (g protein+g carbohydrate) + 37 × (g lipid).

Fatty acid composition

Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GLC-FID)/capillary column based on the ISO 5509 (2000) trans-esterification method. The fatty acid profile was analyzed with a Chrompack CP 9001 chromatograph (Chrompack, Middelburg, Netherlands) equipped with a split-splitless injector, a FID, and a Chrompack CP-9050 autosampler. The temperatures of the injector and detector were 250 °C. Separation was achieved on a 50 m x 0.25 mm i.d. fused silica capillary column coated with a 0.19 μm film of CP-Sil 88 (Chrompack). Helium was used as carrier gas at an internal pressure of 120 kPa. The column temperature was 140 °C, for a 5 min hold, and then programmed to increase to 220 °C at a rate of 4 °C/min and then held for 10 min. The split ratio was 1:50, and the injected volume was 1.2 μL. The results were expressed in relative percentage of each fatty acid, calculated by internal normalization of the chromatographic peak area. Fatty acid identification was made by comparing the relative retention times of fatty acids methyl ester (FAME) peaks from samples with standards. A Supelco (Bellefonte, PA, USA) mixture of 37 FAMEs (standard 47885-U) was used. Some fatty acid isomers were identified with individual standards also purchased from Supelco.

Sugar composition

Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) based on the method used by Harada et al. (2004) with minor modifications. Dried powder (1.0 g) was extracted with 40 mL of 80% aqueous ethanol at 80 ºC for 30 min. The resulting suspension was centrifuged at 15,000*g* for 10 min. The supernatant was concentrated at 60 ºC under reduced pressure and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40 ºC, the solid residues were dissolved in water to a final volume of 5 mL. Soluble sugars were determined by using HPLC (Smartline system, Knauer, Germany) at 35 ºC. The HPLC system was equipped with a Knauer Smartline 2300 RI detector and with a Eurospher 100-5 NH_2 column (4.6 x 250 mm, 5 mm, Knauer). The mobile phase was acetonitrile/deionized water, 7:3 (v/v) at a flow rate of 1.25 mL/min. The results were expressed in g/100 g of fresh weight, calculated by internal normalization of the chromatographic peak area. Sugar identification was made by comparing the relative

retention times of sample peaks with standards. The sugar standards used for identification were purchased from Sigma Chemical Co.: L(+)-arabinose, D(-)-fructose, D(+)-galactose, D(+)-glucose anhydrous, lactose monohydrate, maltose monohydrate, D(+)-mannitol, D(+)-mannose, D(+)-melezitose, D(+)-melibiose monohydrate, L(+)-rhamnose monohydrate, D(+)-sucrose, D(+)-trehalose and D(+)-xylose.

3.1.1.3. Results and discussion

The results of the chemical composition and estimated energetic value obtained for the five mushroom species are shown in **Table 3.1.1**.

Table 3.1.1. Proximate chemical composition (g/100 g of fresh weight) and energetic value of five Portuguese wild edible mushrooms (Mean ± SD; n=3).

	Mushrooms				
	A. arvensis	*L. deliciosus*	*L. giganteus*	*S. imbricatus*	*T. portentosum*
Moisture	94.90±0.75	90.05±0.53	92.43±0.76	93.89±0.00	93.05±0.51
Total fat	0.14±0.00	0.22±0.00	0.41±0.02	0.09±0.01	0.38±0.02
Crude protein	2.87±0.19	2.96±0.04	3.40±0.01	2.35±0.02	2.12±0.08
Ash	0.18±0.01	0.51±0.02	0.65±0.04	0.29±0.08	0.81±0.03
Carbohydrates	1.91±0.24	6.26±0.15	3.11±0.21	3.38±0.03	3.64±0.16
Energy (Kcal)	20.38±1.71	38.86±0.75	29.73±1.05	23.73±0.28	26.46±1.14
Energy (KJ)	86.44±7.27	164.88±3.19	125.84±4.44	100.74±1.18	111.98±4.82

The moisture ranged from 90.05 g/100 g of fresh weight in *L. deliciosus* and 94.90 g/100 g in *A. arvensis*. Protein was found in relatively high levels and varied between 2.12 g/100 g in *T. portentosum* and 3.40 g/100 g in *L. giganteus*. However, it is known that the protein contents of mushrooms are affected by a number of factors, namely the type of mushrooms, the stage of development, the part sampled, the level of nitrogen available and the location (Flegg and Maw, 1977). Fat ranged from 0.09 g/100g in *S. imbricatus* and 0.41 g/100g in *L. giganteus*. This high protein and low fat characteristics of the edible wild mushrooms has been previously reported by many workers (Aletor, 1995; Díez and Alvarez, 2001; Longvah and Deosthale, 1998). Carbohydrates, calculated by difference, were also an abundant macronutrient and ranged from 1.91 g/100 g in *A. arvensis* and 6.26 g/100 g in *L. deliciosus*. Ash content varied between 0.18 g/100 g in *A. arvensis* and 0.81 g/100 g in *T. portentosum*. All the species seem to

have a normal chemical composition compared with other edible mushrooms (Agahar-Murugkar and Subbulakshmi, 2005; Aletor, 1995; Crisan and Sands, 1978; Díez and Alvarez, 2001; Longvah and Deosthale, 1998; Manzi et al., 1999; Manzi et al., 2001; Manzi et al., 2004). On the basis of the proximate analysis, it can be calculated that an edible portion of 100 g of these mushrooms assures, on average, 28 Kcal (118 KJ). The highest values are guaranteed by *L. deliciosus*, while *A. arvensis* give the lowest energy contribution (**Table 3.1.1**).

The results for fatty acid composition, total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) of the studied mushrooms are shown in **Table 3.1.2**.

Table 3.1.2. Fatty acid composition (percent) of five Portuguese wild edible mushrooms Mean ± SD; n=3).

	Mushrooms				
	A. arvensis	*L. deliciosus*	*L. giganteus*	*S. imbricatus*	*T. portentosum*
C14:0	2.34±0.16	0.48±0.00	2.70±0.01	0.27±0.03	0.13±0.00
C15:0	0.79±0.02	0.53±0.05	0.33±0.00	1.22±0.05	0.95±0.01
C16:0	14.55±0.27	12.08±0.06	13.46±0.05	11.14±0.05	5.60±0.01
C16:1c	4.32±0.22	0.92±0.03	12.91±0.05	0.98±0.01	0.51±0.03
C17:0	0.56±0.00	0.18±0.01	0.08±0.00	0.13±0.02	0.05±0.00
C18:0	3.37±0.23	25.33±0.06	2.11±0.02	3.65±0.11	2.33±0.01
C18:1c	15.46±1.67	41.26±0.02	21.09±0.51	45.06±0.20	58.36±0.06
C18:2c	56.11±1.49	17.06±0.23	46.18±0.48	35.38±0.38	30.88±0.08
C18:3c	0.19±0.03	0.26±0.02	0.09±0.02	0.16±0.00	0.40±0.01
C20:0	0.87±0.02	0.44±0.03	0.12±0.00	0.88±0.01	0.13±0.00
C20:1c	0.07±0.02	0.10±0.00	0.07±0.01	0.15±0.01	0.15±0.00
C21:0	0.15±0.05	0.11±0.05	0.07±0.01	0.08±0.01	0.06±0.00
C22:0	0.47±0.04	0.38±0.03	0.12±0.00	0.57±0.01	0.23±0.00
C22:6c	0.37±0.04	0.27±0.09	0.40±0.01	0.20±0.07	0.11±0.02
C24:0	0.37±0.06	0.60±0.06	0.27±0.01	0.13±0.05	0.08±0.02
Total SFA	23.47±0.06	40.14±0.13	19.25±0.02	18.08±0.09	9.57±0.02
total MUFA	19.85±1.48	42.28±0.01	34.08±0.46	46.20±0.21	59.03±0.03
total PUFA	56.68±1.42	17.59±0.12	46.67±0.47	35.73±0.30	31.40±0.05

In general, the major fatty acids found in the studied samples were linoleic acid (C18:2 n-6) and oleic acid (C18:1 n-9), followed by palmitic acid (C16:0). This is in agreement with the results reported for the Indian mushrooms, *Schizophyllum commune* and

Lentinus edodes, in which linoleic (~65%), palmitic (~20%) and oleic (~10%) acids accounted for almost the whole of the fatty acids determined (Longvah and Deosthale, 1998). Similar observations have been made in other mushrooms (Senatore et al., 1988). The fatty acid profile of several *Tricholoma* species was already determined and once more, for *T. portentosum* and *T. terreum*, oleic (~57%) and linoleic (~28%) acid were the main fatty acid constituents, being other fatty acids detected were found only in small amounts (Díez and Alvarez, 2001). It is known that linoleic acid is the precursor of 1-octen-3-ol, known as the alcohol of fungi, which is the principal aromatic compound in most fungi and might contribute to mushrooms flavour (Maga, 1981). Besides the three main fatty acids already described, twelve more were identified and quantified. Some of them were already described, but for different mushroom species and not so exhaustively. PUFA were the main group of fatty acids in *A. arvensis* and *L. giganteus*, while in *L. deliciosus*, *S. imbricatus* and *T. portentosum*, MUFA were the main group. In all cases, UFA predominate over SFA for all the studied mushroom species, ranging from 59.9 to 90.4%. This is consistent with previous observations showing that, in mushrooms, unsaturated fatty acids predominate over the saturated in the total fatty acid content (Díez and Alvarez, 2001; Longvah and Deosthale, 1998; Mauger et al., 2003). Considering total MUFA content, *A. arvensis* had the lowest value but contained the highest PUFA content, also being the mushroom with the highest value of linoleic acid. *T. portentosum* had the lowest SFA value, and the highest value of oleic acid presenting values in agreement with those published previously (Díez and Alvarez, 2001). Fortunately, *trans* isomers of unsaturated fatty acids were not detected in the studied mushrooms. A rapidly expanding literature documents the negative effects of *trans* fatty acids (TFAs) in human health due to the increased risk of cardiovascular disease where they are negatively correlated with plasma HDL-cholesterol concentration and positively correlated with plasma LDL-cholesterol level (Minamide and Hammond, 1985). It is also important to point out that, in contrast to other fungi (Díez and Alvarez, 2001; Longvah and Deosthale, 1998), no other fatty acids with an odd number of carbon atoms have been detected in considerable amounts.

Table 3.1.3. Sugar composition (g/100 g of fresh weight) of five Portuguese wild edible mushrooms (Mean ± SD; n=3).

	Mushrooms				
	A. arvensis	*L. deliciosus*	*L. giganteus*	*S. imbricatus*	*T. portentosum*
Mannitol	0.33±0.00	1.36±0.01	0.14±0.00	1.38±0.01	0.07±0.00
Trehalose	0.02±0.01	0.27±0.01	0.50±0.00	0.51±0.01	1.46±0.03
Total sugars	0.35±0.01	1.63±0.01	0.64±0.00	1.89±0.01	1.53±0.03

In what concerns sugar composition (**Table 3.1.3.**) the five mushrooms showed relative homogeneity. All of them presented mannitol and trehalose as main sugars. For *A. arvensis*, *L. delicisosus* and *S. imbricatus* mannitol was the most abundant sugar ranging from 0.07 to 1.38 g/100 g of fresh weight (**Figure 3.1.1**), while trehalose predominates in *L. giganteus* (0.50 g/100 g) and *T. portentosum* (1.46 g/100 g). The accumulation of these sugars in the fruit-bodies of *Hypsizygus marmoreus* (Peck) Bigelow (Harada et al., 2004), *Agaricus bisporus* Sing. (Minamide and Hammond, 1985), *Flammulina velutipes* (Curt.: Fr.) (Kitamoto and Gruen, 1976) and *Lentinus edodes* (Berk.) Sing. (Yang et al., 2001) has been already reported. With the exception of *A. arvensis*, other sugars were present either only in small amounts or were not detected.

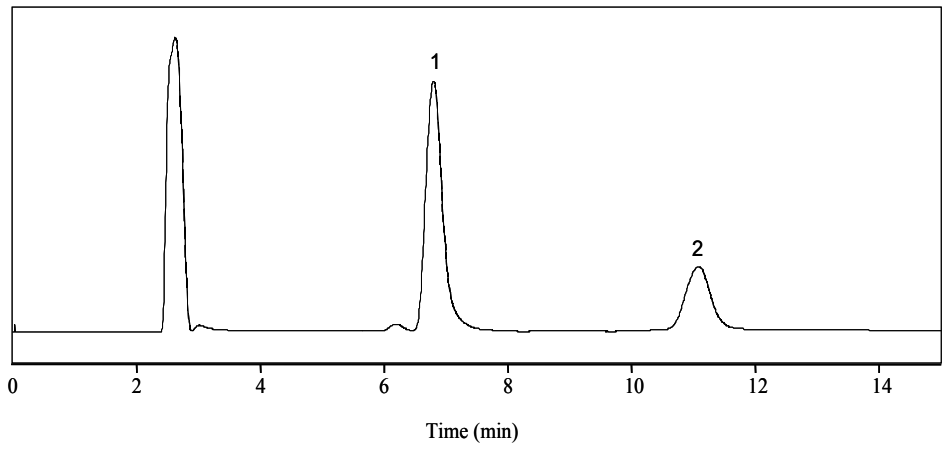


Figure 3.1.1. Individual sugar chromatogram of *Sarcodon imbricatus*. 1- mannitol; 2-trehalose.

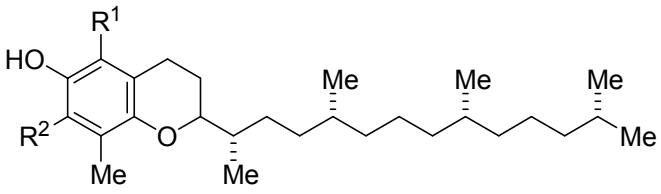
In conclusion, the chemical composition and energy values of the studied Portuguese wild edible mushrooms clearly indicate that they provide key nutrients such as protein, unsaturated fatty acids, and carbohydrates. Being a good source of protein and carbohydrate, and prove to be excellent foods to be used in low caloric diets for their low contents of fat and energy. Nevertheless, the high nutritional quality and unique

flavours of these mushrooms are likely to be lost if these wild edibles are not documented. Therefore, it is now imperative that a nutritional database of these mushrooms is set up to retain the information on these unique species and for a better management and conservation of this natural resource and habitats related to them.

3.1.2. Optimization of the determination of tocopherols in Agaricus sp. edible mushrooms by a normal phase liquid chromatography method

3.1.2.1. Introduction

Tocopherols and tocotrienols are known under the generic name vitamin E, and the chemical name tocochromanols. Tocopherols (**Figure 3.1.2**) are methyl-substituted hydroxychromans with a polar chromanol ring and an apolar phytyl side chain (Kamal-Eldin and Appelqvist, 1996). There are four tocopherol vitamers (α-, β-, γ- and δ-tocopherol) which can be distinguished by the number and location of methyl groups on their chromanol ring (Ryynanen et al., 2004).



R^1=R^2=Me α-tocopherol
R^1=Me, R^2=H β-tocopherol
R^1=H, R^2=Me γ-tocopherol
R^1=R^2=H δ-tocopherol

Figure 3.1.2. Chemical structures of the four tocopherol isomers.

For healthy people it is relatively easy to obtain sufficient tocopherols and tocotrienols from the diet to prevent well-defined vitamin E deficiency symptoms. Most plant-derived foods, especially fruits and vegetables, contain low to moderate levels of vitamin E activity; but, due to the abundance of plant-derived foods in our diets, they provide a significant and consistent source of vitamin E (Chun et al., 2006). Furthermore, high intakes of E vitamers may decrease the risk of several chronic diseases related to oxidative damage, e.g., coronary heart diseases and cancer. Most of these effects (in the human body) are associated to their action as lipid-soluble antioxidants (Stampfer and Rimm, 1995; Stone and Papas, 1997; Theriault et al., 1999; Schwenke, 2002). The biological activities of these compounds are mainly attributed to their antioxidant activity in inhibiting lipid peroxidation in biological membranes. They

act as antioxidant by donating a hydrogen atom to peroxyl radicals of unsaturated lipid molecules, forming a hydroperoxide and a tocopheroxyl radical, which reacts with other peroxyl or tocopheroxyl radicals forming more stable adducts (Lampi et al., 1999). In fact, vitamin E plays a fundamental role in the prevention of radical formation in biological systems like plasma, membranes, tissues, and in the free radical chain reactions interruption (Sanagi et al., 2005).

In the past, α-tocopherol was the most studied vitamer and it was reported to exhibit the highest biological activity. Thus, several methods were developed exclusively for the determination of this compound. However, many studies focusing on the health effects of the other vitamin E isoforms have also been published (Campbell et al., 2003; Mishima et al., 2003).

There is a growing interest in natural antioxidants for their potential role in the prevention of oxidative stress-related diseases, since synthetic antioxidants are being questioned due to their potential carcinogenic activity (Fukushima and Tsuda, 1985). Therefore, natural antioxidants such as tocopherol, polyphenols and carotenoid pigments are having a greater relevance in the protection against lipid oxidation.

The use of mushrooms extracts as antioxidants is becoming increasingly popular (Lo and Cheung, 2005; Turkoglu et al., 2007) and could bring diverse physiological benefits to the consumer, such as protection against human diseases associated with oxidative stress, like coronary heart disease and cancer (Halliwell and Gutteridge, 1999; Lindequist, 2005). Accordingly, there is a great need for an efficient technique for separating and quantifying the individual antioxidant components such as tocopherols. Although various reports have been published on the tocopherols content of mushrooms (Mau et al., 2002a; Mau et al., 2002b; Yang et al., 2002; Elmastas et al., 2007; Tsai et al., 2007), all reported the same methodology including saponification and do not present validation assays. In this study, an extraction method that did not include saponification has been selected, and an antioxidant used to avoid tocopherols oxidation, with special precautions to protect the samples from light and heat. The accuracy, precision and robustness of the method were improved by incorporating an internal standard (IS) from the extraction step through to the chromatographic analysis. As well, the use of fluorescence rather than UV as the detection mode provided the sensitivity and the selectivity required for the accurate determination of low levels of these homologues in mushrooms.

3.1.2.2. Materials and methods

Standards and reagents

Tocopherol standards (α, β, γ and δ) were purchased from Sigma Chemical Co. (St. Louis, MO). Butylated hydroxytoluene (BHT) was obtained from Merck (Darmstadt, Germany), n-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Lab-Scan (Lisbon, Portugal). All other reagents were of analytical grade.

Preparation of standard solutions

Individual stock solutions (~5 mg/mL) of α, β, and γ tocopherols were prepared in hexane and stored protected from light, at -20ºC. A stock standard mixture with the three isomers was prepared in hexane with the final concentration of 1 mg/mL for each isomer. Working standard mixture with concentration of 10 μg/mL was prepared from the stock standard solution. δ-Tocopherol was used as internal standard (IS), being prepared a stock solution at 50 μg/mL in hexane, kept at -20ºC, protected from light, and diluted to a working solution (1.6 μg/mL). BHT was prepared in hexane at a concentration of 10 mg/mL. **Figure 3.1.3** shows a chromatogram of a standards mixture.

Samples

Five mushrooms species: *Agaricus bisporus* (Lange) Imbach, *Agaricus arvensis* Schaeffer, *Agaricus romagnesii* Wasser, *Agaricus silvaticus* Schaeff, and *Agaricus silvicola* (Vittadini) Peck were analysed. The first was a commercial sample obtained in a local supermarket and was used during method development and validation procedures. All the others were wild species and were collected under glassland in Bragança (Northeast of Portugal), in autumn 2006. Taxonomic identification was made according to several authors (Moser, 1983; Courtecuisse and Duhem, 1995) and representative voucher specimens were deposited at the herbarium of *Escola Superior Agrária* of *Instituto Politécnico de Bragança*. After collection and taxonomic identification, all the mushrooms were dried in a liophylizator (Ly-8-FM-ULE, Snijders, Holland) before analysis.

Extraction procedure

BHT solution (100 μL) and IS solution (250 μL) were added to the sample prior to the extraction procedure. The samples (~500 mg) were homogenized with methanol (4 mL) by vortex mixing (1 min). Subsequently, hexane (4 mL) was added and again vortex mixed for 1 min. After that, saturated NaCl aqueous solution (2 mL) was added, the mixture was homogenized (1 min), centrifuged (5 min, 4,000*g*) and the clear upper layer was carefully transferred to a vial. The sample was re-extracted twice with hexane. The combined extracts were taken to dryness under a nitrogen stream, redissolved in 1 mL of n-hexane, dehydrated with anhydrous sodium sulphate, filtered through a 0.22 μm disposable LC filter disk, transferred into a dark injection vial and analysed by HPLC.

HPLC analysis

The HPLC equipment consisted of an integrated system with a Smartline pump 1000 (Knauer, Germany), a degasser system Smartline manager 5000, an AS-2057 auto-sampler and a 2500 UV detector at 295 nm (Knauer) connected in series with a FP-2020 fluorescence detector (Jasco, Japan) programmed for excitation at 290 nm and emission at 330 nm. Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Polyamide II (250 x 4.6 mm) normal-phase column from YMC Waters (Japan) operating at 30ºC (7971 R Grace oven). The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min, and the injection volume was 20 μL. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response, using the internal standard method. Tocopherol contents in mushroom samples were expressed in ng per g of dry mushroom.

Validation assays

The repeatability and reproducibility of the chromatographic method were evaluated using standard solutions of the four vitamers at the concentration levels 80, 400 and 800 ng/mL. For the repeatability, the standards solutions were analysed seven times in the same day, while for the reproducibility the standards solutions were analysed 3 times in 5 consecutive days.

Also, the precision of the extraction method was validated taking into account their repeatability, reproducibility, precision and accuracy. The repeatability and reproducibility was accomplished using a mushroom sample, *Agaricus bisporus*, by

analyzing three times in the same day and in five consecutive days, respectively. Precision was assessed by three extractions of the same mushroom sample being each one analyzed in five consecutive days. The accuracy of the method was evaluated by the standard addition procedure (% of recovery), with three addition levels (80, 400 and 800 ng/mL) each one in triplicate. The standards mixture was added to the sample, and all the extraction procedures were carried out.

Statistical analysis

For each one of the *Agaricus* sp. three samples were analysed and also all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The mushrooms tocopherols content was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SAS v. 9.1.3 program.

3.1.2.3. Results and discussion

Chromatographic conditions

It is know that NP-HPLC (normal-phase) is generally preferred instead of RP-HPLC (reversed-phase) for tocopherols determination since the last system does not completely resolve β- and γ- isomers. Beside, NP-HPLC has the advantage of allowing the use of organic solvents, thus achieving higher lipid solubility and higher loading capacity (Kamal-Eldin et al., 2000; Amaral et al., 2005). For the separation of vitamin E isomers, the YMC-Pack Polyamine II NP-column and hexane/ethyl acetate as mobile phase were adopted. The coefficients of variation for the retention time achieved in this work were low (less then 0.5 % for all standard compounds) (**Table 3.1.4**).

The quantification was performed by the internal standard method in order to improve accuracy, precision and robustness characteristics. The isomer δ-tocopherol was chosen as the internal standard (I.S.; **Figure 3.1.3B**) because it was not detected in the analyzed mushrooms, as also presented an adequate recovery. The recovery of the I.S. was obtained dividing the area of δ-tocopherol standard (equivalent to 400 ng/mL) by the area of the spiked sample after adding δ-tocopherol to obtain a final concentration of 400 ng/mL. The I.S. concentration was kept constant in all the analyses. Therefore, the concentration of each tocopherol found in the mushroom samples is obtained using the response factor for each tocopherol, calculated from the calibration, multiplied by the

concentration of the I.S. and by the response of the isomer present in the sample divided by the response of the I.S. in the same injection (quantification by the internal standard method using DataApex 2.4 software).

A fluorescence detector was selected for the quantification purpose since it provided a higher sensitivity than the UV detector. When the contents of the compounds to quantify are low, it is imperative to use a more sensitive detector as fluorescence to achieve more satisfactory results. In order to allow the detection of the four isomers, the best conditions achieved were: 290 nm for excitation and 330 nm for emission, gain 100.

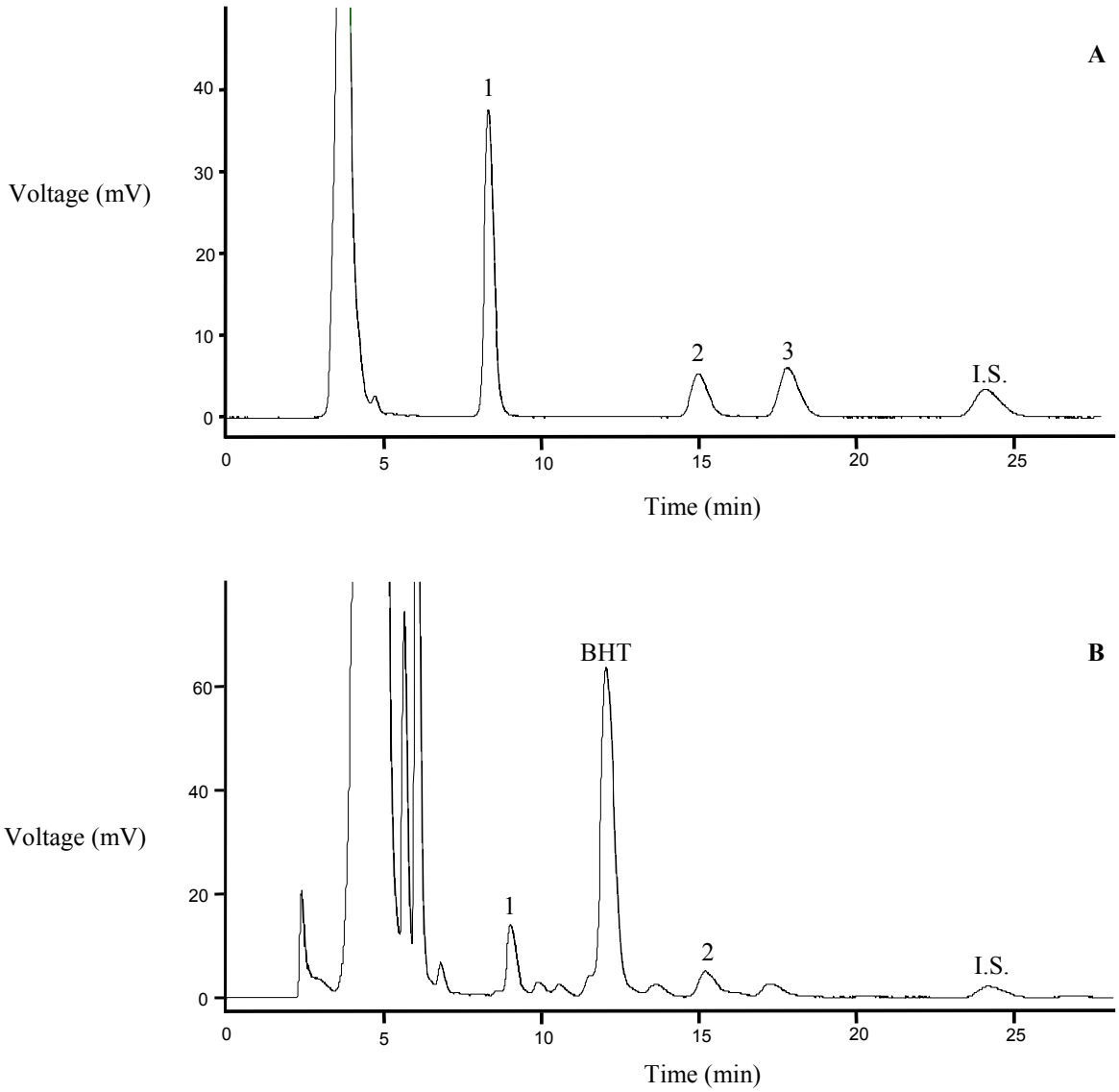


Figure 3.1.3. HPLC fluorescence chromatograms of the working standards mixture (A) and of a mushroom sample extracted using method III (B). Peaks: 1- α-tocopherol; 2- β-tocopherol; 3- γ-tocopherol; I.S.- internal standard (δ-tocopherol); BHT- butylated hydroxytoluene.

Table 3.1.4. Retention times, linearity and sensitivity of HPLC analysis.

Standard	R_t (retention time)		Correlation coefficient (r^2)	Linearity range (ng/mL)	Limits	
	min	CV, % (n=11)			LOD (ng/mL)	LOQ (ng/mL)
α-tocopherol	8.75	0.31	0.9999	40.0-800.0	8.49	28.29
β-tocopherol	16.07	0.43	0.9997	80.0-800.0	20.03	66.77
γ-tocopherol	19.23	0.48	0.9997	80.0-800.0	20.08	66.93
δ-tocopherol	26.18	0.53	I.S.	I.S.	I.S.	I.S.

I.S.- Internal Standard; LOD- Limit of detection ; LOQ- Limit of quantification.

Linearity and sensitivity of HPLC analysis

Table 3.1.4 presents the linearity range, limits of detection (LOD) and limits of quantification (LOQ) determined in the present work. After studying the linearity for each compound (11 levels), a 7-level calibration curve was constructed using the peak-area ratio between the three vitamin E isomers and I.S. *versus* concentration ratio between the standards and I.S. This calibration curve was injected in five different days and the average obtained was used. The correlation coefficient was always higher than 0.999 for all the compounds.

The limits of detection were calculated as the concentration corresponding to three times the calibration error divided by the slope (Ribani et al., 2004), and varied from 8.49 to 20.08 ng/mL. The limits of quantification were calculated using the concentration corresponding to ten times the calibration error divided by the slope (Ribani et al., 2004), and ranged from 28.29 to 66.93 ng/mL.

Validation assays

For the repeatability of the chromatographic method, standards solutions (80, 400 and 800 ng/mL) were analysed seven times in the same day, while for the reproducibility the standards solutions were analysed 3 times in 5 consecutive days. The percentage variation coefficients (CV %) obtained for the response factor are shown in **Table 3.1.5**. The chromatographic method proved to be precise for all the isomers being the obtained values very low (CV% between 0.22 and 0.91%).

Table 3.1.5. Repeatability and reproducibility for the response factor of each standard solution in the HPLC analysis.

Standard	Repeatability CV, % (n=7)			Reproducibility CV, % (n=15)		
	S_1	S_2	S_3	S_1	S_2	S_3
α-tocopherol	0.51	0.65	0.29	0.64	2.72	0.36
β-tocopherol	0.69	0.63	1.04	0.97	4.63	2.29
γ-tocopherol	0.22	0.59	0.94	0.84	3.50	2.04

Standards solutions (S_1 – 80 μg/mL; S_2 – 400 μg/mL; S_3 – 800 μg/mL).

Table 3.1.6 presents the CV% obtained in the validation assays of the extraction methodology.

Table 3.1.6. Method validation parameters obtained using a commercial *Agaricus bisporus* sample.

Compound	Content (ng /g, dry matter)	Repeatability CV% (n=3)	Reproducibility CV% (n=15)	Precision[a] CV% (n=15)
α-tocopherol	749 ± 21	2.95	5.02	6.40
β-tocopherol	1659 ± 4	0.27	2.58	2.35
γ-tocopherol	*nd*.	*nd*.	*nd*.	*nd*.
δ-tocopherol	I.S.	I.S.	I.S.	I.S.

nd.- not detected. [a] CV% of the results obtained after injection of three sample extractions in five consecutive days.

The method used for the mushroom sample proved to be precise and all the values obtained were low (CV% between 0.27 and 2.95% for repeatability and between 2.58 and 5.02% for reproducibility). The CV% for precision, ranged from 2.35 to 6.40%. The results demonstrated recovery percentages between 88.3 and 113.7%, being the lower percentage obtained for β-tocopherol (**Table 3.1.7**). From analysis of **Table 3.1.7** it can be observed that the recovery percentage is lower for the samples spiked with lower concentration levels; nevertheless, all the values obtained are statistically acceptable.

Table 3.1.7. Recovery percentages obtained after addition to *Agaricus bisporus* sample of different levels of concentration of each tocopherol and further analysis in triplicate.

	Accuracy (Recovery, %)			
	Added concentration (ng/mL)			
	80	400	800	Mean
α-tocopherol	105.1 ± 1.1	115.3 ± 0.5	120.1 ± 1.0	113.7 ± 7.6
β-tocopherol	79.1 ± 0.8	90.5 ± 0.9	96.3 ± 0.6	88.3 ± 8.7
γ-tocopherol	97.7 ± 0.3	112.1 ± 1.0	119.4 ± 0.8	109.7 ± 11
δ-tocopherol	-	99.2 ± 0.2	-	99.2 ± 0.2

Tocopherols content in mushroom samples

Using this methodology, α- and β-tocopherol were identified and quantified, while δ- , γ-tocopherol, and tocotrienols were not detected in the analysed mushroom samples.

The tocopherols content in the five *Agaricus* species samples from Northeast Portugal is presented in **Table 3.1.8**. In all the samples β-tocopherol was the major compound, ranging from 677 to 1,931 ng/g. α-Tocopherol was found in all the species with the exception of *A. romagnesii*, while δ- and γ-tocopherols were not detected in any of the samples. The results obtained in the analysis of mushroom samples point to the existence of apparent

differences in what concerns tocopherols composition among different species. *A. silvaticus* presented the higher content of tocopherols (3,232 ng/g) while *A. silvicola* revealed the lower content (1,164 ng/g).

Table 3.1.8. Tocopherols content (ng/g of dry weight) in mushroom samples. In each row different letters mean significant differences ($p<0.05$).

	A.arvensis	*A.bisporus*	*A. romagnesii*	*A.silvaticus*	*A. silvicola*
α-tocopherol	70 ± 2 d	749 ± 21 b	*nd*.	1301 ± 13 a	487 ± 34 c
β-tocopherol	1148 ± 34 d	1659 ± 4 b	1289 ± 15 c	1931 ± 10 a	677 ± 48 e
Total tocopherols	1218 ± 37 c	2409 ± 21 b	1289 ± 15 c	3232 ± 3 a	1164 ± 82 c
Dry matter (%)	5.1 ± 0.8 c	8.2 ± 0.4 b	12.5 ± 0.9 a	7.5 ± 0.6 b	5.1 ± 0.1 c

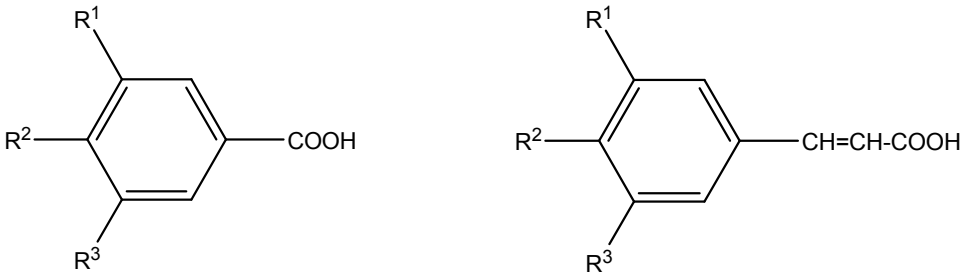
nd.- not detected

Overall, as far as we know, this is the first study reporting an optimization of the determination of tocopherols in mushrooms. It describes a new extraction methodology without saponification step, new chromatographic conditions including fluorescence detection, and simultaneous detection of α and β-tocopherol isomers. The results obtained demonstrate that the proposed method is sensitive, precise, and accurate allowing the determination of tocopherols.

3.1.3. Phenolic acids determination by HPLC-DAD-ESI/MS in sixteen different Portuguese wild mushrooms species

3.1.3.1. Introduction

Natural phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups and can range from simple molecules (phenolic acids, phenylpropanoids, flavonoids) to highly polymerised compounds (lignins, melanins, tannins) (Bravo, 1998). Particularly, phenolic acids can be subdivided into two major groups, hydroxybenzoic acids and hydroxycinnamic acids (**Figure 3.1.4**).



Hydroxybenzoic acids Cinnamic acids derivatives

Figure 3.1.4. Chemical structure of the identified phenolic acids in the wild mushroom species. Benzoic acids: *p*-hidroxybenzoic (R^1=R^3=H, R^2=OH), protocatechuic (R^1=H, R^2=R^3=OH), vanillic (R^1=CH_3O, R^2=OH, R^3=H). Cinnamic acid (R^1=R^2=R^3=H) and derivatives: *p*-coumaric (R^1=R^3=H, R^2=OH).

Hydroxybenzoic acids include *p*-hydroxybenzoic, protocatechuic, vanillic, syringic, and gallic acids. They are commonly present in the bound form and are typically a component of a complex structure like lignins and hydrolyzable tannins. They can also be found linked to sugar derivatives and organic acids in plant foods. Hydroxycinnamic acids include *p*-coumaric, caffeic, ferulic, and sinapic acids. In natural sources they are mainly found esterified with small molecules like, e.g., quinic or tartaric acids, as well as bound to cell-wall structural components such as cellulose, lignin, and proteins through ester bonds (Liu, 2004).

Phenolic compounds constitute one of the most numerous and ubiquitously distributed group of plant secondary metabolites, with more than 8000 phenolic structures currently known (Andersen and Markam, 2006). These compounds are synthesized by higher plants probably as a result of antioxidative strategies adapted in evolution by respirative

organisms starting from precursors of cyanobacteria. They also provide essential functions in the reproduction and the growth of the plants being capable of a wide range of responses to genetic and environmental influences, acting as defence mechanisms against pathogens, parasites, and predators, as well as contributing to the colour of plants (Signore et al., 1997; Bennick, 2002; Apak et al., 2007). In addition to their roles in plants, phenolic compounds, commonly found in vegetables, fruits and many plant-derived foods that form a significant portion of our diet, are among the most potent and therapeutically useful bioactive substances, providing health benefits associated with reduced risk of chronic and degenerative diseases (Luximon-Ramma et al., 2003; Luximon-Ramma et al., 2005; Soobrattee et al., 2005). Many phenolic compounds have been reported to exhibit a wide range of biological effects including antibacterial, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic and vasodilatory actions (Middleton et al., 2000); many of these biological functions have been attributed to their free radical scavenging and antioxidant activity.

Although the dietary intake of phenolics varies considerably among geographic regions, it is estimated that daily intake range from about 20 mg to 1 g, much higher than that for Vitamin E (Hollman and Katan, 1998).

The use of mushrooms extracts as antioxidants is becoming increasingly popular (Mau et al., 2002b; Lo and Cheung, 2005; Elmastas et al., 2007; Tsai et al., 2007), however, little is known about the individual phenolic compounds present in mushroom species. A few studies concerning the analysis of the phenolic components of Portuguese wild mushrooms can be found in the literature, particularly for *Cantharellus cibarius* (Valentão et al., 2005), *Suillus bellini*, *Tricholomopsis rutilans*, *Hygrophorus agathosmus*, *Amanita rubescens*, *Russula cyanoxantha*, *Boletus edulis*, *Tricholoma equestre*, *Suillus luteus*, *Suillus granulatus* (Ribeiro et al., 2006), and *Fistulina hepatica* (Ribeiro et al., 2007). Nevertheless, being the Northeast of Portugal one of the European regions with higher wild edible mushroom diversity, it is important to characterize the phenolic composition of other species also important and with gastronomic relevance. In this study, for the first time, individual profiles of phenolic compounds in sixteen Portuguese wild mushrooms were characterised by high-performance liquid chromatography coupled to photodiode array detector and mass spectrometer (HPLC-DAD-ESI/MS).

3.1.3.2. Materials and methods

Samples

Sixteen mushrooms species were collected from different places in Trás-os-Montes region in the Northeast of Portugal (**Table 3.1.9)**. The morphological identification of the wild macrofungi was made till species according to macro and microscopic characteristics, and following several authors (Moser, 1983; Courtecuisse and Duhem, 1995) and representative voucher specimens were deposited at the herbarium of *Escola Superior Agrária* of *Instituto Politécnico de Bragança*. After taxonomic identification, the mushrooms were immediately lyophilized (Ly-8-FM-ULE, Snijders, Holland), and kept in the dark in hermetically sealed plastic bags up to analysis.

Table 3.1.9. Collection information of mushroom samples.

Species	Origin	Orchard	Date of collection
Agaricus arvensis	Carrazeda de Ansiães	*Pinus pinaster*	October 2006
Agaricus bisporus	Bragança	Grassland	October 2006
Agaricus romagnesii	Vinhais	*Pinus pinaster*	October 2006
Agaricus silvícola	Bragança	*Quercus pyrenaica*	October 2006
Cantharellus cibarius	Vinhais	*Quercus pyrenaica*	June 2007
Hypholoma fasciculare	Bragança	*Quercus pyrenaica*	October 2006
Lactarius deliciosus	Bragança	*Pinus pinaster*	November 2005
Lactarius piperatus	Bragança	*Quercus pyrenaica*	June 2006
Lepista nuda	Cova de Lua	*Pinus pinaster*	November 2006
Leucopaxillus giganteus	Cova de Lua	*Pinus pinaster*	October 2005
Lycoperdon molle	Vinhais	*Quercus pyrenaica*	October 2006
Lycoperdon perlatum	Vinhais	*Quercus pyrenaica*	October 2006
Macrolepiota procera	Carrazeda de Ansiães	*Quercus pyrenaica*	November 2006
Ramaria botrytis	Vinhais	*Quercus pyrenaica*	October 2006
Sarcodon imbricatus	Vinhais	*Pinus pinaster*	November 2006
Tricholoma acerbum	Vinhais	*Quercus pyrenaica*	October 2006

Standards and reagents

Acetonitrile 99.9% was of HPLC grade from Lab-Scan (Lisbon, Portugal). All the other reagents (methanol, n-hexane, ethyl acetate and diethyl ether) were of analytical grade purity and were also supplied by Lab-Scan. Gallic acid was from Supelco (Bellefonte, PA, USA) and the rest of phenolic standards were from Sigma Chemical Co. (St. Louis, MO, USA). The Folin and Ciocalteu's reagent was purchased from Merck (Darmstadt,

Germany). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

Analysis of total phenolics

Sample preparation. A fine dried mushroom powder (20 mesh) sample (~3 g) was extracted by stirring with 100 mL of methanol at 25 ºC at 150 rpm for 24 h and filtered through Whatman Nº 4 paper. The residue was then extracted with two additional 100 mL portions of methanol, as described earlier. The combined methanolic extracts were evaporated at 40 ºC to dryness and redissolved in a known concentration of methanol.

Folin Ciocalteu's assay. Total phenolic compounds were determined according to the procedure described in **3.1.4.2** (*Total bioactive compounds*).

DPPH radical-scavenging activity was evaluated as described in chapter **3.2.1.2**.

Phenolic compounds identification and quantification

Sample preparation. Each mushroom sample (~3 g) was extracted with acetone:water (80:20; 50 mL) mixture at -20°C for 6h. The extract was put in an ultrasonic bath for 15 min, centrifuged at 4000*g* for 10 min, and filtered through Whatman nº 4 paper. The residue was then extracted with three additional 50 mL portions of the acetone:water mixture. The combined extracts were evaporated at 30 ºC to remove acetone. The aqueous phase was washed with n-hexane, and then submitted to a liquid-liquid extraction with diethyl ether (3 x 50 mL) and ethyl acetate (3 x 50 mL). The organic phases were evaporated at 30 ºC to dryness, redissolved in water:methanol (80:20), and filtered through a 0.22 μm disposable LC filter disk for HPLC analysis.

HPLC-DAD-ESI/MS analyses. The phenolic extracts were analysed using a Hewlett-Packard 1100 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany). Separation was achieved on a Spherisorb (Phenomenex, Torrance, CA) reverse phase C_{18} column (3 μm, 150mm x 4.6mm i.d.) thermostatted at 25 ºC. The solvents used were: (A) 2.5% acetic acid in water, (B) acetic acid 2.5%/acetonitrile (90:10), and (C) 100% HPLC-grade acetonitrile. The gradient employed was: isocratic 100% A for 10 min, 50% A and 50% B for 10 min, isocratic 100% B for 15 min, 90% B and 10% C for 10 min, 70% B and 30% C for 10 min, 50% B and 50% C for 5 min, 20% B and 80% C for 5 min, 100% A for 5 min, at a flow rate of 0.5 mL/min. Detection was carried out in a diode array detector

(DAD), using 280 nm as the preferred wavelength, and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet.

LC–MS analyses were performed using a Finnigan^{TM} LCQ MS detector (Thermoquest, San Jose, CA, USA) equipped with an API source, using an electrospray ionisation (ESI) interface. Both the sheath gas and the auxiliary gas were nitrogen at flow rates of 1.2 and 6 L/min, respectively. The capillary and source voltage were 10V and 3.5 kV, respectively, and the capillary temperature was 175 ºC. Spectra were recorded in negative ion mode between *m/z* 80 and 620. The MS was programmed to carry out a series of three consecutive scans: a full mass from 150 to 1500 amu, a zoom scan of the most abundant ion in a ±5 amu range, and an MS-MS scan of the most abundant ion in the full mass using a normalised energy of collision of 45%.

The phenolic compounds present in the samples were characterised according to their UV-vis spectra and identified by their mass spectra and retention times in comparison with those of commercial standards. For the quantitative analysis of phenolic compounds, a calibration curve was obtained by injection of different concentration of protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, and cinnamic acid standards.

Statistical analysis

The analysis of phenolic compounds contents in each mushroom species was carried out in triplicate and the results expressed as mean ± standard deviation (SD). Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$, using SPSS v. 16.0 program.

3.1.3.3. Results and discussion

Total phenolic compounds in the analysed Portuguese wild mushrooms species were determined by the Folin Ciocalteu's assay. **Table 3.1.10** presents those results as also the EC_{50} values (extract concentration correspondent to 50% of radical scavenging activity) obtained in the assessment of the antioxidant activity of mushrooms measured by the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay (see chapters 3.2.1; 3.2.2; 3.2.4; 3.4.1 and 3.4.2).

Table 3.1.10. Total phenolic compounds (by Folin Ciocalteu's assay) and antioxidant activity (by DPPH assay) of the wild mushrooms. In each column different letters mean significant differences ($p<0.05$).

Species	Total phenols (mg/g extract)	Antioxidant activity (EC_{50} value, mg/mL)
Agaricus arvensis	2.75 ± 0.17 f	15.85 ± 0.27 d
Agaricus bisporus	4.49 ± 0.16 e	9.61 ± 0.07 e
Agaricus romagnesii	6.18 ± 0.44 d	6.22 ± 0.10 gf
Agaricus silvícola	6.40 ± 0.17 d	6.39 ± 0.16 f
Cantharellus cibarius	1.75 ± 0.50 g	19.65 ± 0.28 b
Hypholoma fasciculare	17.67 ± 0.27 b	1.13 ± 0.03 l
Lactarius deliciosus	3.40 ± 0.18 f	16.31 ± 0.24 c
Lactarius piperatus	3.09 ± 0.12 f	20.24 ± 0.78 a
Lepista nuda	6.31 ± 0.13 d	4.41 ± 0.01 i
Leucopaxillus giganteus	6.29 ± 0.20 d	1.44 ± 0.09 l
Lycoperdon molle	11.48 ± 0.52 c	3.23 ± 0.09 k
Lycoperdon perlatum	10.57 ± 0.17 c	3.95 ± 0.04 j
Macrolepiota procera	3.17 ± 0.92 f	5.38 ± 0.50 h
Ramaria botrytis	20.32 ± 1.87 a	0.66 ± 0.00 m
Sarcodon imbricatus	3.06 ± 0.10 f	5.82 ± 0.06 g
Tricholoma acerbum	5.53 ± 0.63 d	3.60 ± 0.08 kj

Phenolic compounds include different subclasses (flavonoids, phenolic acids, stilbenes, lignans, tannins, oxidized polyphenols) displaying a large diversity of structures, some of which may escape the usual methodologies of analysis, commonly carried out by HPLC (High Performance Liquid Chromatography) coupled to distinct detection devices. Various reasons exist for that, like the existence of isomers, difficulty for chromatographic separation of some compounds, lack of commercial standards, or structure not yet elucidated (Georgé et al., 2005). The method of Folin Ciocalteu's is, therefore, largely used to evaluate total phenolics despite all the interferences of this assay since the reagent (mixture of phosphotungstic acid and phosphomolibdic acid) also reacts with other non-phenolic reducing compounds leading to an overvaluation of the phenolic content. For instance, ascorbic acid is a widespread reducing agent that can interfere in the Folin-Ciocalteu reaction (Georgé et al., 2005) and that was, in fact, reported to be present in the studied species (see chapters 3.2.1; 3.2.2; 3.2.4 and 3.4.2). Other reducing substances such

as some sugars and amino acids could also interfere. In addition, the results have to be expressed in equivalents of a particular standard compound (like catechin, gallic acid or tannin acid). All these aspects make the results obtained for different authors difficult to compare.

The antioxidant activity assessed in mushroom extracts by different chemical and biochemical assays has been correlated with their contents of phenolic compounds measured by the Folin-Ciocalteu method. However, no analyses of individual phenolics were made and, therefore, the compounds responsible for that antioxidant activity were unknown. In the present study we aimed to identify and quantify individual compounds that may contribute to the bioactive properties already found for these Portuguese wild mushroom species.

Three phenolic acids (protocatechuic, *p*-hydroxybenzoic and *p*-coumaric acids) and a related compound (cinnamic acid) could be positively identified and quantified in some samples (**Table 3.1.11**) by comparison of their chromatographic characteristics and absorption spectra with the standards compounds and confirmed by mass analysis.

Table 3.1.11. Phenolic acids found in the mushroom samples. In each column different letters mean significant differences ($p<0.05$).

	Phenolic compounds (mg/kg, dry matter)						Other compounds (mg/kg, dry matter)
	protocatechuic acid (15.1 min)	*p*-hydroxybenzoic acid (22.9 min)	vanillic acid isomer (40.5 min)	*p*-coumaric acid (41.7 min)	vanillic acid isomer (44.1 min)	Total phenolic compounds	Cinnamic acid (51.4 min)
A. arvensis	*nd*	70.13 ± 1.20	*nd*	48.67 ± 3.40	*nd*	118.8 ± 4.6 c	49.10 ± 8.03
A. bisporus	*nd*	25.59 ± 1.55	*nd*	*nd*	*nd*	25.59 ± 1.55 e	8.72 ± 0.71
A. silvivola	*nd*	238. 7 ± 12.4	*nd*	45.72 ± 1.19	*nd*	284.4 ± 11.2 b	68.37 ± 11.32
A. romagnesii	*nd*	32.40 ± 0.83	*nd*	*nd*	*nd*	32.40 ± 0.83 e	49.22 ± 3.90
C. cibarius	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	14.97 ± 0.40
L. deliciosus	*nd*	22.66 ± 0.36	*nd*	*nd*	*nd*	22.66 ± 0.36 e	*nd*
L. giganteus	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
L. nuda	33.47 ± 0.50	29.31 ± 1.54	*nd*	3.75 ± 0.56	*nd*	66.53 ± 2.62 d	*nd*
L. molle	*nd*	41.66 ± 0.33	35.97 ± 6.16	*nd*	4.02 ± 0.55	81.65 ± 7.04 d	*nd*
L. perlatum	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	14.36 ± 1.27
L. piperatus	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
M. procera	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	21.53 ± 1.65
H. fascicular	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
S. imbricatus	*nd*	33.19 ± 1.92	*nd*	*nd*	*nd*	33.19 ± 1.92e	*nd*
R. botrytis	342.7 ± 10.2	14.00 ± 0.77	*nd*	*nd*	*nd*	356.7 ± 9.4 a	*nd*
T. acerbum	*nd*	29.66 ± 0.26	4.92 ± 0.72	*nd*	7.81 ± 0.56	42.38 ± 1.53 e	*nd*

nd- not detected

In **Figure 3.1.5** a representative chromatogram obtained for one of the mushroom extracts analysed is shown as an example.

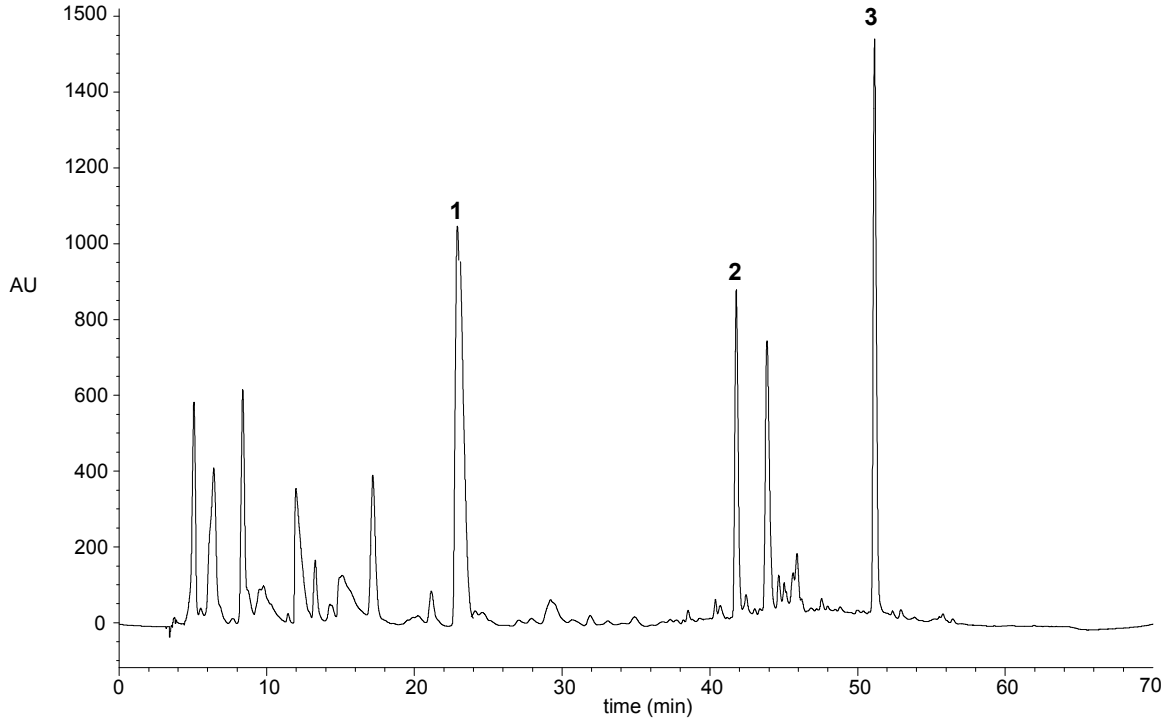


Figure 3.1.5. HPLC chromatogram recorded at 280 nm of an extract of Agaricus silvicola. Only peaks corresponding to phenolic compounds or related compounds are indicated: (1) 2- *p*-hydroxybenzoic acid, (2) 2- *p*-coumaric acid, and (3) cinnamic acid.

Other two compounds were also detected in the samples of *L. molle* and *T. acerbum* whose UV spectra, molecular ion (*m/z* $[M-H]^-$ at 167) and MS^2 spectra (one fragment at *m/z* 123, $[M-44]^-$, loss of a CO_2 residue) coincided with those of vanillic acid (4-hydroxy-3-methoxybenzoic ***acid)***, but that showed higher retention times (40.5 and 44.0 min, respectively, in comparison with 30.1 min for vanillic acid). Thus, these compounds were tentatively associated to vanillic acid isomers like, e.g., *o*-vanillic (i.e., 2-hydroxy-3-methoxy-benzoic acid*)* or isovanillic acid (i.e., 3-hydroxy-4-*methoxybenzoic* acid), for which no standards were available. No phenolic acids were detected in six mushroom species: *H. fasciculare*, *L. piperatus, L. giganteus*, *C. cibarius, L. perlatum* and *M. procera*, although the presence of cinnamic acid was found in the three latter.

No peaks were found in any mushroom extract whose UV spectra could be associated to hydroxycinnamic acids or their derivatives such as tartaric or quinic esters (i.e., chlorogenic acids). Further, no detection of those compounds was made when the full

mass chromatograms of the samples were screened for their molecular ions. Similarly, no peaks whose UV spectra or mass characteristics could be associated to flavonoids were found. This fact should not be surprising since, in general, it is assumed that only plants possess the biosynthetic ability to produce flavonoids and not animals and fungi (Iwashina, 2000), even if some flavonoids have exceptionally been reported from fungi *Aspergillus candidus* and *Phallus impudicus* (reviewed in ref. Iwashina, 2000) and more recently in the edible beefsteak fungus *Fistulina hepatica* (Ribeiro et al., 2007).

Thus, the phenolic composition of the mushrooms seems to be characterised by only the presence of phenolic acids, being *p*-hydroxybenzoic acid the major compound in most cases; among the species analysed, only *L. nuda* and *R. botrytis* showed protocatechuic acid as the main phenolic compound. Other authors had already reported the presence of *p*-hydroxybenzoic acid in other mushroom species, such as *A. rubescens*, *T. equestre* and *R. cyanoxantha* (Ribeiro et al., 2006). The same research group reported the presence of *p*-coumaric acid in *C. cibarius* (Valentão et al., 2005) and *F. hepatica* (Ribeiro et al., 2007). However, we could not find *p*-coumaric acid in our *Cantharellus cibarius* sample.

Ramaria botrytis showed the highest phenolic acids concentration (356.7 mg/Kg, dry matter) mostly due to the contribution of protocatechuic acid (342.7 mg/Kg, dry matter). In fact, this mushroom species also revealed the highest content in total phenolics as determined by the Folin-Ciocalteu assay (**Table 3.1.10**) and the highest antioxidant capacity (lower EC_{50} values). A low correlation between the total phenolics and phenolic acids content was obtained ($Y=0.0318X+ 3.6087$; $R^2=0.4900$), which suggest that other compounds different than phenolic acids are present in mushrooms and react with the Folin-Ciocalteu reagent and also contribute to their antioxidant properties.

As far as we know, this is the first report concerning the phenolic acids composition of *Agaricus arvensis, Agaricus bisporus, Agaricus romagnesii, Agaricus silvicola, Cantharellus cibarius, Hypholoma fasciculare, Lactarius deliciosus, Lactarius piperatus, Lepista nuda, Leucopaxillus giganteus, Lycoperdon molle, Lycoperdon perlatum, Macrolepiota procera, Ramaria botrytis, Sarcodon imbricatus* and *Tricholoma acerbum.* This study also suggests that phenolic acids analysis could be useful in taxonomic studies involving mushroom species, besides their importance as antioxidants for the human health. Nevertheless, further studies are required to conclude about this point, as it is known that the levels of phenolic compounds depend on several factors such as cultivation techniques, cultivar, growing conditions, ripening process,

processing and storage conditions, as well as stress conditions such as UV radiation, infection by pathogens and parasites, wounding air pollution and exposure to extreme temperatures (Naczk, Shahidi, 2006).

3.1.4. Wild and commercial mushrooms as source of nutrients and nutraceuticals

3.1.4.1. Introduction

A nutraceutical can be defined as a product that may be considered a food or part of a food and provides medical or health benefits like the prevention and treatment of disease. Nutraceuticals may range from isolated compounds and dietary supplements to genetically engineered "designer" foods, herbal products and processed products such as cereals, soups and beverages. Some examples of nutritive nutraceuticals or "functional food ingredients" are dietary fiber, polyunsaturated fatty acids (PUFA, fish oil), some proteins, peptides, amino acids and keto acids, some minerals, and antioxidative vitamins and other antioxidants (glutathione, selenium, etc) (Andlauer and Fürst, 2002; Kruger and Mann, 2003). PUFAs, especially the n-3 fatty acid family, are claimed to exert a protective effect against the development of cardiovascular and inflammatory diseases (Fürst and Kuhn, 2000; Fang et al., 2002). Epidemiological studies have consistently shown an inverse association between consumption of vegetables and fruits and the risk of cardiovascular diseases (Bazzano et al., 2001) and certain forms of cancer (Liu, 2004). Although the protective effects have been primarily attributed to well-known antioxidants, such as ascorbic acid, tocopherols and β-carotene, plant phenolics may also play a significant role (Soobrattee et al., 2005).

Different mushrooms were studied by the scientific community in searching for new therapeutic alternatives, and the results proved their bioactive properties (Lindequist et al., 2005). Mushrooms are rich sources of nutraceuticals (Çağlarirmak, 2007; Elmastas et al., 2007; Ribeiro et al., 2007) responsible for their antioxidant (Mau et al., 2002b; Lo and Cheung, 2005), antitumor (Wasser and Weis, 1999a), and antimicrobial properties (Smania et al., 1995, Hirasawa et al., 1999, Hatvani et al., 2001, Turkoglu et al., 2007). Besides their pharmacological features (Lindequist et al., 2005), wild mushrooms are becoming more important in our diet due to their nutritional value, related to the high protein and low fat/energy contents (Díez and Alvarez, 2001, Agahar-Murugkar et al., 2005).

In the present study we intend to evaluate the composition of wild and commercial mushrooms in nutrients and nutraceuticals. The evaluation of nutrient composition included the determination of proteins, fats, ash, carbohydrates, and individual profile of

sugars. The evaluation of nutraceutical composition included the determination of fatty acids, phenolics, flavonoids, carotenoids, ascorbic acid and tocopherols. Antimicrobial activity was screened against fungi, Gram positive and Gram negative bacteria, and correlated to the bioactive compounds present in the extracts.

3.1.4.2. Materials and methods

Samples

Eight mushrooms species: *Boletus edulis* Fr., *Calocybe gambosa* (Fr.) Donk, *Cantharellus cibarius* L. ex Fr., *Craterellus cornucopioides* Pers., *Marasmius oreades* (Bolt. ex Fr.) Fr. were commercial dried samples obtained in several supermarkets. All the others (*Agaricus bisporus* (Lange) Imbach, *Agaricus silvaticus* Schaeff., *Agaricus silvicola* (Vittad.) Peck were wild species were collected under grassland in Bragança (Northeast of Portugal), in autumn 2006. The morphological identification of the wild macrofungi was made till species according to macro and microscopic characteristics, and following several authors (Moser, 1983; Courtecuisse and Duhem, 1995) and representative voucher specimens were deposited at the herbarium of *Escola Superior Agrária* of *Instituto Politécnico de Bragança*. After collection and taxonomic identification, those mushrooms were lyophilized (Ly-8-FM-ULE, Snijders, Holland) before analysis.

Standards and reagents

Acetonitrile 99.9%, n-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Lab-Scan (Lisbon, Portugal). All the other reagents were of analytical grade purity: methanol and diethyl ether were supplied by Lab-Scan; toluene from Riedel-de-Haën (Hanover, Germany); sulphuric acid from Fluka (St. Gallen, Switzerland). The fatty acids methyl ester (FAME) reference standard mixture 37 (fatty acids C4 to C24; (standard 47885-U) was from Supelco (Bellefonte, PA, USA) and from Sigma Chemical Co. (St. Louis, MO, USA), as also other individual fatty acid isomers, tocopherol standards (α, β, γ and δ), and the standards gallic acid and (+)-catechin. The standards used in the antimicrobial activity assays, ampicillin and cycloheximide, as also butylated hydroxytoluene (BHT) were purchased from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma. Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

Nutrient composition

Nutritional value. Samples of mushrooms were analysed for chemical composition (protein, fat, carbohydrates and ash) using the procedures described in **3.1.1.2** (C*hemical composition*). Reducing sugars were determined by DNS (dinitrosalicylic acid) method.

Sugar composition. Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) based on the procedure described in **3.1.1.2** (*Sugar composition*).

Nutraceutical composition

Fatty acid composition. Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GLC-FID)/capillary column based on the following trans-esterification procedure: fatty acids were methylated with 5 mL of methanol:sulphuric acid:toluene 2:1:1 (v:v), during at least 12 h in a bath at 50 ºC and 160 rpm; then 5 mL of deionised water were added, to obtain phase separation; the FAME were recovered with 5 mL of diethyl ether by shaking in vortex , and the upper phase was passed through a micro-column of sodium sulphate anhydrous, in order to eliminate the water; the sample was recovered in a vial with Teflon, and before injection the sample was filtered with 0.2 µm nylon filter from Milipore (Massachusetts, USA). The fatty acid profile was analyzed with a DANI model GC 1000 instrument (DANI Instruments S.p.A., Milan, Italy) equipped with a split/splitless injector, a flame ionization detector (FID) and a Macherey-Nagel column (30 m x 0.32 mm ID x 0.25 µm d_f). The oven temperature program was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 10ºC/min ramp to 240 ºC and held for 11 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC. For each analysis 1 µL of the sample was injected in GC. Fatty acid identification was made by comparing the relative retention times from samples with FAME peaks (standards). The results were recorded and processed using CSW 1.7 software (DataApex 1.7) and expressed in relative percentage of each fatty acid.

Tocopherol composition. Quantification was based on the procedure described in **3.1.2.2** (*Extraction procedure* and *HPLC analysis*). Tocopherol contents in mushroom samples are expressed in μg per g of dry mushroom.

Total bioactive compounds. Bioactive compounds (phenolics, flavonoids, ascorbic acid and carotenoids) were determined after methanolic extraction of the samples.

Phenolic compounds in the mushroom extracts were estimated by a colorimetric assay, based on procedures described by Singleton and Rossi (1965) with some modifications. Briefly, 1 mL of sample was mixed with 1 mL of Folin and Ciocalteu's phenol reagent. After 3 min, 1 mL of saturated sodium carbonate solution was added to the mixture and adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm (Analytikijena 200-2004 spectrophotometer, Jena, Germany). Gallic acid was used to calculate the standard curve (0.01-0.4 mM; Y=2.8557X-0.0021; R^2=0.9999) and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

Flavonoid contents in the extracts were determined by a colorimetric method described by Jia et al. (1999) with some modifications. The mushroom extract (250 μL) was mixed with 1.25 mL of distilled water and 75 μL of a 5% $NaNO_2$ solution. After 5 min, 150 μL of a 10% $AlCl_3.H_2O$ solution was added. After 6 min, 500 μL of 1M NaOH and 275 μL of distilled water were added to the mixture. The solution was mixed well and the intensity of pink colour was measured at 510 nm. (+)-catechin was used to calculate the standard curve (0.022-0.34 mM; Y=0.9629X-0.0002; R^2=0.9999) and the results were expressed as mg of (+)-catechin equivalents (CEs) per g of extract.

Ascorbic acid was determined according to the method of Klein and Perry (1982). The dried methanolic extract (100 mg) was extracted with 10 mL of 1% metaphosphoric acid for 45 min at room temperature and filtered through Whatman No. 4 filter paper. The filtrate (1 mL) was mixed with 9 mL of 2,6-dichlorophenolindophenol and the absorbance was measured within 30 min at 515 nm against a blank. Content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid (0.020–0.12 mg/mL; Y=3.4127X-0.0072; R^2=0.9905) and the results were expressed as mg of ascorbic acid/g of extract.

β-Carotene and lycopene were determined according to the method of Nagata and Yamashita (1992). The dried methanolic extract (100 mg) was vigorously shaken with 10 mL of acetone–hexane mixture (4:6) for 1 min and filtered through Whatman No. 4

filter paper. The absorbance of the filtrate was measured at 453, 505 and 663 nm. Contents of β-carotene and lycopene were calculated according to the following equations: lycopene (mg/100 mL) = - 0.0458 A663 + 0.372 A505 - 0.0806 A453; β-carotene (mg/100 mL) = 0.216 A663 - 0.304 A505 + 0.452 A453. The results were expressed as μg of carotenoid/g of extract.

Antimicrobial activity

The assays were performed in the methanolic extracts after redissolution in DMSO at a concentration of 50 mg/mL.

Microorganisms and culture conditions. Microorganisms labeled CECT were obtained from the Spanish type culture collection (CECT) of Valencia University, while microorganisms labeled ESA were clinically isolated strains from different biological fluids, identified by Microbiology Laboratory of Escola Superior Agrária de Bragança. Gram + (*Bacillus cereus* CECT 148, *B. subtilis* CECT 498, *Staphylococus aureus* ESA 7 isolated from pus) and Gram – (*Escherichia coli* CECT 101, *Pseudomonas aeruginosa* CECT 108, *Klebsiella pneumoniae* ESA 8 isolated from urine) bacteria, and fungi (*Candida albicans* CECT 1394, *Cryptococcus neoformans* ESA 3 isolated from vaginal fluid) were used to screen samples antimicrobial activity. Microorganisms were cultured aerobically at 37 ºC (Scientific 222 model) in nutrient agar medium for bacteria, and at 30 ºC in sabouraud dextrose agar medium for fungi.

Test assays for antimicrobial activity. A screening of antibacterial activities against the Gram - and Gram + bacteria and fungi was performed, and the minimal inhibitory concentration (MIC) was determined by an adaptation of the agar streak dilution method based on radial diffusion (Hawkey and Lewis, 1994; Ferreira et al., 2004). Suspensions of the microorganisms were prepared to contain approximately 10^8 cfu/mL, and the plates containing agar medium were inoculated (100 μL). A 50 μL volume of each sample was placed in a hole (depth 3 mm, diameter 4 mm) made in the centre of the agar. Under the same conditions, different solutions of ampicillin (antibacterial) and cycloheximide (antifungal) were used as standards. The assays with the standards were carried out using also DMSO solutions, which was chosen as the best solvent. After comparative toxicity assays this solvent showed to be not toxic. The MIC was

considered to be the lowest concentration of the tested sample able to inhibit the growth of bacteria or fungi, after 24h. The diameters of the inhibition zones corresponding to the MICs were measured using a ruler, with an accuracy of 0.5 mm. Each inhibition zone diameter was measured three times (three different plates) and the average was considered. A control using only inoculation was also carried out.

Statistical analysis

For each one of the mushroom species three samples were analysed and also all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$, using SAS v. 9.1.3 program.

3.1.4.3. Results and discussion

Nutrient composition

The results of the chemical composition and estimated energetic value (expressed on dry weight basis) of the wild and commercial edible mushrooms are shown in **Table 3.1.12**. Protein was found in high levels and varied between 17.18 g/100 g in *B. edulis* and 80.93 g/100 g in *A. bisporus*. Fat ranged from 0.92 g/100g in *A. bisporus* and 4.88 g/100g in *C. cornucopioides*. In general, wild mushrooms were richer sources of protein and had a lower amount of fat than commercial mushrooms. Carbohydrates, calculated by difference, were also an abundant macronutrient and ranged from 8.25 g/100 g in *A. bisporus* and 71.15 g/100 g in *B. edulis*. Reducing sugars are only a small part of carbohydrates content, being polysaccharides such as chitin and starch the most abundant carbohydrates in mushrooms. Ash content varied between 7.07 g/100 g in *B. edulis* and 16.48 g/100 g in *A. silvaticus*. Wild mushroom species proved to be less energetic providing, on average, 1,502 KJ per 100 g of a dry portion, when compared to the 1,597 KJ assured by the commercial sp. The highest energetic value was obtained for the commercial *B. edulis*, while the wild *A. silvaticus* gave the lowest energetic contribution (**Table 3.1.12**).

Table 3.1.12. Proximate chemical composition (g/100 g) and energetic value (KJ/100 g) of wild and commercial mushrooms (mean ± SD; n=3).

Results are expressed in a dry weight basis. In each column different letters mean significant differences ($p<0.05$).

Specie	Total fat	Crude protein	Ash	Carbohydrates	Reducing sugars	Energy
Agaricus bisporus	0.92 ± 0.06 d	80.93 ± 3.53 a	9.90 ± 0.05 d	8.25 ± 1.42 d	1.44 ± 0.02 cb	1550.05 ± 21.65 c
Agaricus silvaticus	2.05 ± 0.01 c	71.99 ± 4.59 b	16.48 ± 0.23 a	9.49 ± 2.84 d	2.36 ± 0.15 cb	1460.87 ± 23.88 e
Agaricus silvícola	2.43 ± 0.22 cb	70.47 ± 2.01 b	14.93 ± 0.66 b	12.18 ± 2.02 d	2.00 ± 0.08 ed	1494.82 ± 13.89 d
Boletus edulis	4.60 ± 0.13 a	17.18 ± 0.92 e	7.07 ± 0.59 e	71.15 ± 1.55 a	3.39 ± 0.30 a	1671.83 ± 18.72 a
Calocybe gambosa	1.05 ± 0.10 d	47.22 ± 1.32 d	8.72 ± 0.81d	43.01 ± 1.31 b	1.26 ± 0.06 ed	1566.23 ± 11.07 b
Cantharellus cibarius	4.49 ± 0.06 a	69.14 ± 3.26 b	12.12 ± 0.26 c	14.25 ± 3.96 d	2.54 ± 0.16 cb	1572.87 ± 22.63 cb
Craterellus cornucopioides	4.88 ± 020 a	69.45 ± 1.24 b	12.22 ± 0.81 c	13.44 ± 1.85 d	2.70 ± 0.29 b	1583.78 ± 14.28 b
Marasmius oreades	2.99 ± 0.82 b	52.22 ± 2.57 c	11.39 ± 0.09 c	29.41 ± 3.32 c	2.21 ± 0.33 cb	1589.91 ± 12.07 cb

The edible mushrooms contained mannitol and trehalose as the principal carbohydrates (**Table 3.1.13**).

Table 3.1.13. Sugar composition (g/100 g) of wild and commercial mushrooms (mean ± SD; n=3). Results are expressed in a dry weight basis. In each column different letters mean significant differences ($p<0.05$).

	Mannitol	Trehalose	Maltose	Melezitose	Total sugars
Agaricus bisporus	19.57 ± 0.07 a	0.77 ± 0.01 e	*nd*	0.53 ± 0.00 b	20.87 ± 0.07 a
Agaricus silvaticus	2.65 ± 0.02 f	0.25 ± 0.15 fg	0.44 ± 0.02 b	0.38 ± 0.05 cb	3.72 ± 0.10 g
Agaricus silvícola	6.09 ± 0.44 d	0.66 ± 0.07 fe	0.57 ± 0.03 a	0.47 ± 0.01 cb	7.79 ± 0.54 f
Boletus edulis	3.46 ± 0.19 e	9.71 ± 0.19 b	*nd*	0.29 ± 0.01 d	13.46 ± 0.37 c
Calocybe gambosa	0.27 ± 0.00 g	8.01 ± 0.38 c	*nd*	0.85 ± 0.11 a	9.13 ± 0.48 e
Cantharellus cibarius	8.33 ± 0.09 c	6.12 ± 0.10 d	*nd*	*nd*	14.45 ± 0.19 b
Craterellus cornucopioides	10.67 ± 0.20 b	0.11 ± 0.01 g	*nd*	*nd*	10.78 ± 0.21 d
Marasmius oreades	2.42 ± 0.09 f	10.49 ± 0.02 a	*nd*	0.55 ± 0.04 b	13.46 ± 0.03 c

nd- not detected

The accumulation of these sugars in the fruit-bodies of other species was already reported (Harada et al., 2004) and also observed by us (see chapter 3.1.1). Nevertheless, the present study also describes the presence of maltose (disaccharide) and melezitose (non-reducing trisaccharide) in some of the studied species. *A. bisporus* revealed the highest sugar contents (20.87 g/100 g), while *A. silvaticus* revealed the lowest levels (3.72 g/100g).With the exception of *A. bisporus*, the commercial species seem to have higher concentration of sugars. Otherwise, maltose was not found in none of the commercial mushrooms. This sugar can be broken down into two glucose molecules by hydrolysis, and the drying process to which commercial species were submitted could be responsible for the degradation of maltose. Melezitose is obtained especially from exudations of various trees or from honey made from such exudations, and therefore incorporated by some mushrooms.

Nutraceutical composition

The results of fatty acid composition, total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) of the studied mushrooms are shown in **Table 3.1.14**.

Table 3.1.14. Fatty acid composition (percent) of wild and commercial mushrooms (mean ± SD; n=3). In each row different letters mean significant differences ($p<0.05$).

	A. bisporus	*A. silvaticus*	*A. silvicola*	*B. edulis*	*C. gambosa*	*C. cibarius*	*C. cornucopioides*	*M. oreades*
C6:0	0.03 ± 0.00	0.03 ± 0.00	0.06 ± 0.01	0.02 ± 0.00	0.40 ± 0.07	0.67 ± 0.05	0.04 ± 0.00	0.19 ± 0.02
C8:0	0.03 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.08 ± 0.00	0.12 ± 0.03	0.04 ± 0.00	0.13 ± 0.01	0.05 ± 0.01
C10:0	0.05 ± 0.00	0.07 ± 0.00	0.06 ± 0.00	0.01 ± 0.00	0.44 ± 0.04	0.01 ± 0.00	0.07 ± 0.00	0.02 ± 0.00
C12:0	0.05 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.19 ± 0.01	0.03 ± 0.00	0.07 ± 0.01	0.07 ± 0.00
C14:0	0.49 ± 0.02	0.32 ± 0.01	0.30 ± 0.02	0.15 ± 0.00	0.40 ± 0.03	0.13 ± 0.00	0.07 ± 0.01	0.15 ± 0.04
C15:0	1.03 ± 0.02	0.65 ± 0.01	0.85 ± 0.07	0.21 ± 0.00	0.65 ± 0.06	0.31 ± 0.02	0.22 ± 0.00	0.60 ± 0.03
C16:0	9.97 ± 0.03	11.74 ± 0.08	9.96 ± 0.20	10.03 ± 0.28	15.16 ± 1.34	13.08 ± 0.14	6.66 ± 0.29	13.82 ± 0.26
C16:1	0.14 ± 0.02	0.76 ± 0.01	0.58 ± 0.04	0.53 ± 0.00	0.88 ± 0.08	0.49 ± 0.03	0.18 ± 0.01	1.00 ± 0.09
C17:0	0.68 ± 0.01	0.39 ± 0.00	0.92 ± 0.01	0.15 ± 0.00	0.20 ± 0.02	0.19 ± 0.01	0.13 ± 0.00	0.07 ± 0.02
C18:0	4.08 ± 0.10	1.41 ± 0.00	2.64 ± 0.20	2.75 ± 0.06	2.14 ± 0.27	6.49 ± 0.11	7.83 ± 0.40	1.65 ± 0.22
C18:1n9c	1.30 ± 0.05	6.67 ± 0.01	3.49 ± 0.20	39.72 ± 1.64	18.10 ± 0.68	10.78 ± 0.34	51.85 ± 0.45	28.52 ± 1.72
C18:2n6c	75.72 ± 0.15	74.78 ± 0.01	76.50 ± 0.21	44.32 ± 2.02	57.75 ± 0.98	53.59 ± 0.12	23.67 ± 1.37	50.66 ± 0.63
C18:3n3	0.06 ± 0.00	0.10 ± 0.00	0.05 ± 0.00	0.07 ± 0.01	0.45 ± 0.07	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
C20:0	2.13 ± 0.01	0.85 ± 0.01	1.77 ± 0.00	0.44 ± 0.02	0.33 ± 0.02	0.31 ± 0.02	0.32 ± 0.02	0.30 ± 0.03
C20:1c	0.07 ± 0.01	0.13 ± 0.00	0.12 ± 0.00	0.49 ± 0.03	*nd*	11.48 ± 0.02	7.57 ± 0.05	*nd*
C20:2c	0.21 ± 0.01	0.15 ± 0.00	*nd*	0.14 ± 0.00	0.12 ± 0.01	0.17 ± 0.00	0.05 ± 0.01	0.20 ± 0.01
C20:3n3+C21:0	0.42 ± 0.03	0.21 ± 0.03	0.36 ± 0.00	0.03 ±0.00	0.12 ± 0.03	0.23 ± 0.02	*nd*	0.03 ± 0.00
C22:0	1.62 ± 0.00	0.81 ± 0.03	1.30 ± 0.18	0.30 ± 0.01	0.59 ± 0.05	0.38 ± 0.03	0.35 ± 0.05	0.59 ± 0.05
C23:0	0.37 ± 0.01	0.21 ± 0.03	0.15 ± 0.01	0.04 ± 0.00	0.87 ± 0.06	0.11 ± 0.01	0.05 ± 0.00	0.22 ± 0.02
C24:0	1.56 ± 0.02	0.55 ± 0.03	0.69 ± 0.01	0.31 ± 0.00	0.99 ± 0.03	0.88 ± 0.06	0.43 ± 0.02	1.16 ± 0.08
C24:1	*Nd*	0.11 ± 0.01	0.05 ± 0.00	0.17 ± 0.00	0.07 ± 0.01	0.55 ± 0.04	0.24 ± 0.00	0.65 ± 0.05
SFA	**22.08 ± 0.19 a**	**17. 10 ± 0.01 c**	**18.80 ± 0.46 b**	**14.52 ± 0.33 d**	**22.51 ± 0.54 a**	**22.63 ± 0.24 a**	**16.36 ± 0.86 c**	**18.88 ± 0.86 b**
MUFA	**1.52 ± 0.06 h**	**7.67 ± 0.02 f**	**4.25 ± 0.24 g**	**40. 91 ± 1.67 b**	**19.05 ± 0.58 e**	**23.29 ± 0.25 d**	**59.85 ± 0.51 a**	**30.16 ± 1.39 c**
PUFA	**76.41 ± 0.13 a**	**75.23 ± 0.02 a**	**76.95 ± 0.22 a**	**44.56 ± 2.01 e**	**58.42 ± 1.12 b**	**54.08 ±0.01 c**	**23.79 ± 1.37 f**	**50.96 ± 0.53 d**

nd- not detected; C- Carbon atoms; SFA- saturated fatty acids; MUFA- monounsaturated fatty acids; PUFA- polyunsaturated fatty acid

In general, the major fatty acid found in the studied species was linoleic acid (C18:2), followed by oleic acid (C18:1) and palmitic acid (C16:0). Besides the three main fatty acids already described, eighteen more were identified and quantified. PUFA were the main group of fatty acids in all species with the exception of *C. cornucopiodes*, where MUFA were the main group. Wild *Agaricus* sp. contained a lower value of MUFA but also a higher content of PUFA than the commercial species, due to the higher contribution of linoleic acid. The commercial *C. gambosa* revealed the highest SFA values, due to the contribution of palmitic acid. Nevertheless, UFA predominated over SFA in all the studied mushrooms, ranging from 77 to 85%. The commercial *B. edulis* contained the highest UFA (85%) and the lowest SFA levels (15%).

Tocopherol contents in the eight studied mushroom, including three wild (chapter **3.1.2**) and five commercial species are presented in **Table 3.1.15**.

Table 3.1.15. Tocopherol composition (μg/g) of the wild and commercial mushrooms (mean ± SD; n=3). Results are expressed in a dry weight basis. In each column different letters mean significant differences ($p<0.05$).

	α-tocopherol	β-tocopherol	γ-tocopherol	Total
Agaricus bisporus[a]	0.75 ± 0.04 b	1.66 ± 0.01 b	*nd*	2.41 ± 0.05cb
Agaricus silvaticus[a]	0.49 ± 0.03c	0.68 ±0.05 c	*nd*	1.16 ± 0.08 ed
Agaricus silvicola[a]	1.30 ± 0.01 a	1.93 ± 0.01 b	*nd*	3.23 ± 0.00 b
Boletus edulis	0.32 ± 0.04 d	8.90 ± 0.77 a	1.42 ± 0.07 a	10.65 ± 0.84 a
Calocybe gambosa	0.06 ± 0.00 f	0.20 ± 0.02 c	0.14 ± 0.02 c	0.41 ± 0.01 ef
Cantharellus cibarius	0.12 ± 0.00 f	0.03 ± 0.01 c	0.03 ± 0.00 d	0.18 ± 0.01 f
Craterellus cornucopioides	0.24 ± 0.02 e	1.55 ± 0.05 b	0.08 ± 0.01 dc	1.87 ± 0.02 cd
Marasmius oreades	0.06 ± 0.00 f	0.19 ± 0.09 c	1.30 ± 0.09 b	1.55 ± 0.05 d

[a] Chapter **3.1.2**; *nd*.- not detected

α and β-Tocopherol were found in all mushroom species, either wild or commercial. The first isomer was found in higher amounts in the wild species (results also shown in **Table 3.1.8**, section ***3.1.2.3***), while significant differences were not observed in β-tocopherol content of wild and commercial sp. γ-Tocopherol was only found in the commercial species.

Table 3.1.16 presents phenolics, flavonoids, ascorbic acid and carotenoids concentrations obtained in the extracts from commercial mushrooms. Phenols were the major antioxidant component detected in the extracts (0.88-8.94 mg/g), followed by flavonoids (0.67-3.40 mg/g). Ascorbic acid was found in small amounts (0.03–0.87

mg/g), and β-carotene and lycopene were only found in vestigial levels (<0.01 mg/g). Wild species (results also shown in **Table 3.2.4**, section ***3.2.2.3***) revealed a higher content in phenols, but a lower content in ascorbic acid than commercial mushrooms.

Table 3.1.16. Total bioactive compounds of wild and commercial mushrooms (mean ± SD; n=3). Results are expressed per g of extract. In each column different letters mean significant differences ($p<0.05$).

	Phenolics (mg/g)	Flavonoids (mg/g)	Ascorbic acid (mg/g)	β-carotene (μg/g)	Lycopene (μg/g)
Agaricus bisporus[a]	4.49 ± 0.16 d	1.73 ± 0.11 d	0.03 ± 0.00 c	1.95 ± 0.10 g	0.91 ± 0.06 eb
Agaricus silvaticus[a]	8.94 ± 0.44 a	3.40 ± 0.01 a	0.04 ± 0.00 c	5.42 ± 0.10 d	2.63 ± 0.06 c
Agaricus silvicola[a]	6.18 ± 0.44 b	2.87 ± 0.12 b	0.04 ± 0.00 c	3.02 ± 0.12 e	2.63 ± 0.06 d
Boletus edulis	5.03 ± 0.11 c	1.75 ± 0.13 d	*nd*	2.73 ± 0.32 fe	1.14 ± 0.08 b
Calocybe gambosa	1.70 ± 0.10 g	1.18 ± 0.16 e	0.40 ± 0.01 b	6.41 ± 1.27 c	3.30 ± 0.88 b
Cantharellus cibarius	0.88 ± 0.09 h	0.67 ± 0.11 f	0.86 ± 0.01 a	13.56 ± 0.51 a	5.06 ± 0.33 a
Craterellus cornucopioides	2.13 ± 0.38 f	1.71 ± 0.29 d	0.87 ± 0.02 a	12.77 ± 0.19 b	5.13 ± 0.14 a
Marasmius oreades	3.20 ± 0.30 e	2.26 ± 0.18 c	*nd*	1.99 ± 0.14 fg	0.54 ± 0.08 e

[a] Chapter **3.2.2**.; *nd*.- not detected

Antimicrobial activity

Table 3.1.17 shows the antimicrobial screening of mushroom extracts against *B. cereus*, *B. subtilis*, *S. aureus* (Gram +), *E. coli*, *P. aeruginosa*, *K. pneumoniae* (Gram -) bacteria, and *C. albicans* and *C. neoformans* (fungi). *A. silvaticus*, *C. cornucopioides*, *M. oreades* and *C. gambosa* did not reveal antimicrobial properties, at the tested concentrations; the other species showed antimicrobial activity selectively against Gram + bacteria, with very low MICs.

C. cibarius and *A. bisporus* revealed MIC values against *B. subtilis* even lower than the standard ampicillin. Likewise, *B. edulis* and *A. silvicola* revealed MIC values against *S. aureus* lower than the standard. The antimicrobial activity was not dependent of being commercial or wild mushroom, since *A. bisporus* and *A. silvicola* of the wild sp., and *B. edulis* and *C. cibarius* of the commercial sp. showed antimicrobial efficiency; it certainly depends on antimicrobials present in each mushroom species.

Table 3.1.17. Antimicrobial activity of wild and commercial mushrooms (mean ± SD; n=3).

	MIC (μg/mL)							
Samples	*B. cereus*	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*E. coli*	*K. peumoniae*	*C. albicans*	*C. neoformans*
Agaricus bisporus	500 (-)	5 (++++)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)
Agaricus silvaticus	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)
Agaricus silvicola	5 (++++)	50 (++++)	5 (++++)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)
Boletus edulis	500 (-)	500 (-)	5 (++++)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)
Calocybe gambosa	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)
Cantharellus cibarius	500 (-)	5 (++++)	50 (++++)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)
Craterellus cornucopioides	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)
Marasmius oreades	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)
Ampicillin	3.13 (++++)	12.5 (++++)	6.25 (++++)	6.25 (++++)	6.25 (++++)	6.25 (++++)	NT	NT
Cycloheximide	NT	NT	NT	NT	NT	NT	12.5 (++)	6.25 (++++)

No antimicrobial activity at the higher tested concentration (-), inhibition zone < 1 mm. Slight antimicrobial activity (+), inhibition zone 2-3 mm. Moderate antimicrobial activity (++), inhibition zone 4-5 mm. High antimicrobial activity (+++), inhibition zone 6-9 mm. Strong antimicrobial activity (++++), inhibition zone > 9 mm. Standard deviation ± 0.5 mm. NT. not tested.

Among all the studied species, only the chemical composition of *A. bisporus* (Manzi et al., 2001; Krbavcic and Baric 2003), *B. edulis* (Manzi et al., 2004) and *C. cibarius* (Agahar-Murugkar and Subbulakshmi, 2005) was described previously but from different countries. Similar to our samples, the Indian *C. cibarius* contained protein as the principal macronutrient, while in the Italian *B. edulis*, carbohydrates dominated. The Italian *A. bisporus* sample contained higher levels of carbohydrate than proteins, while the opposite was observed in the Portuguese sample. Generally, high protein and carbohydrate contents, and low fat levels were also described by previous authors (Díez and Alvarez, 2001). Nevertheless, the differences between the nutrient concentrations of Portuguese and Indian *C. cibarius* samples and between the Portuguese and Italian *B. edulis* and *A. bisporus* may be attributed to a number of factors, such as mushroom strain/type, composition of growth media, time of harvest, management techniques, handling conditions, and the preparation of the substrates (Manzi et al., 2001). The wild mushroom species proved to be less energetic than the commercial samples with a higher content of protein and a lower fat concentration.

UFA predominated over SFA in all the studied mushrooms, which is consistent with other studies (Díez and Alvarez, 2001). The commercial *B. edulis* contained the highest UFA contents and the lowest SFA levels, leading to an increase in HDL cholesterol and decrease in LDL cholesterol, triacylglycerol, lipid oxidation, and LDL susceptibility to oxidation (Kanu et al., 2007). The same species revealed the highest contents of tocopherols. Some authors published tocopherol determination in *A. bisporus* from Turkey (Elmastas et al., 2007) and *B. edulis* from Taiwan (Tsai et al., 2007), but using a different methodology and presenting the results per mg of extracts. In general, the commercial mushrooms revealed higher concentrations of sugars, and the wild species contained lower values of MUFA but also higher contents of PUFA. α-Tocopherol was detected in higher amounts in the wild species, and γ-tocopherol was not found in these mushrooms. Wild samples showed a higher content in phenols, but a lower content in ascorbic acid than commercial mushrooms.

There were no differences between the antimicrobial properties of wild and commercial mushrooms. The entire extracts were used to measure potential health benefits in order to take advantage of the possible additive and synergistic effects of all the bioactive compounds present. Future studies should be done in order to conclude the mechanism of action involved in antimicrobial growth inhibition; there might be other compounds,

besides those quantified in this study, which contribute to antimicrobial properties of the wild and commercial species.

Recently, the widely consumed *Tricholoma flavovirens* caused delayed rhabdomyolysis in twelve humans from France and Poland, and also in mice after administration of consecutive meals of extracts of this mushroom (Bedry et al., 2001). The investigation was extended, first to *Boletus edulis* (Nieminen et al., 2005) and then to other species such as *Cantharellus cibarius*, *Russula* spp, *Leccinum versipelle*, *Albatrellus ovinus*, (Nieminen et al., 2006). The results indicated elevations in the plasma creatine kinase activities and additional effects on the liver transaminases and plasma creatinine in experimental mice. There has also been a concern about the safety of *A. bisporus*, an extensively consumed mushroom worldwide. Some authors demonstrated that lifetime administration of uncooked *A. bisporus* to mice induced tumours at a number of sites. However, when air-dried mushroom were fed to rats for 500 days, no carcinogenic effect was evident (Walton et al., 1998). Other authors suggested that diets high in *A. bisporus* may modulate the aromatase activity and function in chemoprevention in postmenopausal women by reducing the *in situ* production of estrogen (Grube et al., 2001). However, these findings should be interpreted carefully since these wild mushrooms have been considered safe for millennia. The harmful effects require prolonged daily exposure and high amounts of ingested mushroom (Nieminen et al., 2005).

The analyzed mushrooms contain very useful nutraceuticals such as UFA, phenolics, tocopherols, ascorbic acid, and carotenoids which could be extracted for the purpose of being used as functional ingredients namely against microbial infections. Public health authorities consider prevention and treatment with nutraceuticals a powerful instrument in maintaining and promoting health, longevity and life quality. The beneficial effects of nutraceuticals will undoubtedly have an impact on nutritional therapy; they also represent a growing segment of today's food industry. Besides, these mushrooms might be used directly in diet and promote health, taking advantage of the additive and synergistic effects of all the bioactive compounds present.

3.2. Antioxidant and antimicrobial properties of Portuguese wild mushrooms

3.2.1. Total phenols, ascorbic acid, β-carotene and lycopene in Portuguese wild edible mushrooms and their antioxidant activities

3.2.1.1. Introduction

Free radical formation is associated with the normal natural metabolism of aerobic cells. The oxygen consumption inherent to cell growth leads to the generation of a series of oxygen free radicals. The interaction of theses species with molecules of a lipidic nature produces new radicals: hydroperoxides and different peroxides (Aust and Sringen, 1982; Pryor et al., 1982; Torel et al., 1986. This group of radicals (superoxide, hydroxyl and lipoid peroxides) may interact with biological systems in a clearly cytotoxic manner. In this respect, flavonoids and phenols have been shown to posses an important antioxidant activity towards these radicals, which is principally based on the redox properties of their phenolic hydroxyl groups and the structural relationships between different parts of their chemical structure (Bors and Saran, 1987; Visioli et al., 1998a; Visioli et al., 1998b). Free radicals and their uncontrolled production, in fact, are responsible for several pathological processes, such as certain tumours (prostate and colon cancers) (Keys, 1995) and coronary heart disease (Lipworth et al., 1997).

In the past few years, the suspected toxicity of some synthetic compounds used in food has raised the interest in natural products (Fukushima and Tsuda, 1985; Stone et al., 2003). Some industries, such as those related to food additive production, cosmetics, and pharmaceuticals, have increased their efforts in preparing bioactive compounds from natural products by extraction and purification. Antioxidant compounds can scavenge free radicals and increase shelf life by retarding the process of lipid peroxidation, which is one of the major reasons for deterioration of food products during processing and storage (Halliwell, 1997; Halliwell and Gutteridge, 1999). Thus a need for identifying alternative natural and safe sources of food antioxidants has been created, and the search for natural antioxidants, especially of plant origin, has notably increased in recent years (Skerget et al., 2005).

Vegetables and fruits are rich sources of antioxidants such as vitamin C, vitamin E, carotenoids, polyphenolic compounds and flavonoids (Diplock et al., 1998), which prevent free radical damage reducing risk of chronic diseases. Thus, the consumption of dietary antioxidants from these sources is beneficial in preventing cardiovascular diseases, especially atherosclerosis (Hu, 2000).

Mushrooms have been used for traditional foods and medicines in Asia (Chang, 1996). Mushrooms contain various polyphenolic compounds recognized as an excellent antioxidant due to their ability to scavenge free radicals by single-electron transfer (Hirano, 2001). Some common edible mushrooms, which are widely consumed in Asian culture, have currently been found to possess antioxidant activity which is well correlated with their total phenolic content (Yen and Hung, 2000; Mau et al., 2002a; Yang et al., 2002; Cheung et al., 2003; Mau et al., 2004; Cheung and Cheung, 2005; Lo and Cheung, 2005).

Recently, we carried out the first study on the antioxidant activity of Portuguese wild edible mushrooms (*Lactarius deliciosus* and *Tricholoma portentosum*), comparing the entire mushroom, the cap and stipe DPPH scavenging capacities and reducing powers (Ferreira et al., 2007). Herein, we report the antioxidant activity of three new Portuguese wild edible mushroom species (*Leucopaxillus giganteus, Sarcodon imbricatus, Agaricus arvensis*), and their correlation to the phenols, ascorbic acid, beta-carotene, and lycopene contents. For the screening of mushrooms antioxidant properties we evaluated their reducing power, DPPH radical scavenging activity, inhibition of erythrocytes hemolysis, and we also used the β-carotene linoleate model system.

3.2.1.2. Materials and methods

Standards and Reagents

Standards BHA (2-*tert*-butyl-4-methoxyphenol), TBHQ (tert-butylhydroquinone), L-ascorbic acid, α-tocopherol and gallic acid were purchase from Sigma Chemical Co. (St. Louis, MO, USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals were obtained from Sigma. Methanol was obtained from Pronalab (Lisbon, Portugal). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

Samples

Samples of *L. giganteus* and *A. arvensis* were colleted under grassland whereas *S. imbricatus* was collected under live pine trees (*Pinus* sp.), in Bragança (Northeast of Portugal), in autumn 2005. After collection, the mushrooms were grouped by taxon and were air-dried in a liophylizator (Ly-8-FM-ULE, Snijders, Holland) before analysis. Taxonomic identification was made according to several authors (Marchand, 1971-1986; Moser, 1983; Bon, 1988; Courtecuisse and Duhem, 1995; Courtecuisse, 1999) and representative voucher specimens were deposited at the herbarium of *Escola Superior Agrária* of *Instituto Politécnico de Bragança*.

Sample preparation

A fine dried mushroom powder (20 mesh) sample (5 g) was extracted with methanol in a soxhlet apparatus for 24h. The methanolic extract was evaporated at 40 ºC to dryness and redissolved in methanol at a concentration of 5 mg/ml, and stored at 4 ºC until further use.

Determination of antioxidant components

Phenolic compounds, ascorbic acid, β-carotene and lycopene were determined according to the procedures described in **3.1.4.2** (*Total bioactive compounds*).

Reducing power assay

The reducing power was determined according to the method of Oyaizu (1986). Various concentrations of mushrooms methanolic extracts (2.5 mL) were mixed with 2.5 mL of 200 mmoL/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 ºC for 20 min. After 2.5 mL of 10% tricloroacetic acid (w/v) were added, the mixture was centrifuged at 1000 rpm for 8 min (Centorion K24OR- 2003 refrigerated centrifuge). The upper layer (5 mL) was mixed with 5 mL of deionised water and 1 mL of 0.1% of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm. The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration. BHA and α-tocopherol were used as standards.

DPPH radical-scavenging assay

The capacity to scavenge the "stable" free radical DPPH was monitored according to the method of Hatano et al. (1988). Various concentrations of methanolic extracts from mushrooms (0.3 mL) were mixed with 2.7 mL of methanolic solution containing DPPH radicals ($6x10^{-5}$ mol/L). The mixture was shaken vigorously and left to stand for 60 min in the dark (until stable absorption values were obtained). The reduction of the DPPH radical was determined by measuring the absorption at 517 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations. The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph of RSA percentage against extract concentration. BHA and α-tocopherol were used as standards.

Assay for erythrocyte hemolysis mediated by peroxyl free radicals

The antioxidant activity of the mushrooms methanolic extracts was measured as the inhibition of erythrocyte hemolysis (Miki et al., 1987). Blood was obtained from male ram (churra galega transmontana) of body weight ~67 Kg. Erythrocytes separated from the plasma and the buffy coat were washed three times with 10 mL of 10 mM phosphate buffer saline (PBS) at pH 7.4 (prepared by mixing 10 mM of NaH_2PO_4 and Na_2HPO_4, and 125 mM of NaCl in 1L of distilled water) and centrifuged at 1500 *g* for 5 min. During the last washing, the erythrocytes were obtained by centrifugation at 1500 *g* for 10 min. A 0.1 mL of a 20% suspension of erythrocytes in PBS was added to 0.2 mL of 200 mM 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) solution (in PBS) and 0.1 mL of mushroom methanolic extracts of different concentrations. The reaction mixture was shaken gently (30 rpm) while being incubated at 37 ºC for 3h. The reaction mixture was diluted with 8 mL of PBS and centrifuged at 3000 *g* for 10 min; the absorbance of its supernatant was then read at 540 nm by a spectrophotometer, after filtration with a syringe filter (cellulose membrane 30 mm, 0.20 μm, Titan). The percentage hemolysis inhibition was calculated by the equation % hemolysis inhibition = $[(A_{AAPH}-A_S)/A_{AAPH}] \times 100$, where A_S is the absorbance of the sample containing the

mushroom extract, and A_{AAPH} is the absorbance of the control sample containing no mushroom extract. The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations. The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph of hemolysis inhibition percentage against extract concentration. L-ascorbic acid was used as standard.

Antioxidant assay using the β-carotene linoleate model system

The antioxidant activity of mushroom extracts was evaluated by the β-carotene linoleate model system (Mi-Yae et al., 2003). A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 mL of chloroform. Two millilitres of this solution were pipetted into a 100 mL round-bottom flask. After the chloroform was removed at 40ºC under vacuum, 40 mg of linoleic acid, 400 mg of Tween 80 emulsifier, and 100 mL of distilled water were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing 0.2 mL of different concentrations of the mushroom extracts. The tubes were shaken and incubated at 50ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. Absorbance readings were then recorded at 20-min intervals until the control sample had changed colour. A blank, devoid of β-carotene, was prepared for background subtraction. Antioxidant activity was calculated using the following equation: Antioxidant activity = (β-carotene content after 2h of assay/initial β-carotene content) × 100. The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations. The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated from the graph of antioxidant activity percentage against extract concentration. TBHQ was used as standard.

3.2.1.3. Results and discussion

Determination of antioxidant components

Table 3.2.1 shows the phenols, ascorbic acid, β-carotene, and lycopene concentration in the mushroom extracts. Whereas total phenols were the major antioxidant components found in the mushroom extracts, ascorbic acid was found in small amounts (0.13-0.35 mg/g), and β-carotene and lycopene were only found in vestigial amounts (< 3 μg/g). *L.*

giganteus extracts showed the highest phenolic content (6.29 ± 0.20 mg/g); the amount found in *A. arvensis* extracts (2.83 ± 0.09 mg/g) was slightly lower than the content found in *S. imbricatus* (3.76 ± 0.11 mg/g).

Table 3.2.1. Contents of total phenols, ascorbic acid, β-carotene and lycopene in the mushrooms extracts.

	L. giganteus	*S. imbricatus*	*A. arvensis*
Total phenols (mg/g)	6.29 ± 0.20	3.76 ± 0.11	2.83 ± 0.09
Ascorbic acid (mg/g)	0.13 ± 0.01	0.16 ± 0.01	0.35 ± 0.00
β-carotene (μg/g)	1.88 ± 0.09	2.53 ± 0.11	2.97 ± 0.12
Lycopene (μg/g)	0.69 ± 0.03	1.3 ± 0.07	1.0 ± 0.05

The highest content of total phenols in the *L. giganteus* extracts might account for the better results found in their antioxidant activity. In fact, it had been reported that the antioxidant activity of plant materials is well correlated with the content of their phenolic compounds. Phenolic compounds such as BHT (butylated hydroxytoluene) and gallate are known to be effective antioxidants (Velioglu et al., 1998).

Reducing power assay

Figure 3.2.1 shows the reducing power of mushrooms methanolic extracts as a function of their concentration. In this assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers (i.e., antioxidants) causes the conversion of the Fe^{3+}/ferricyanide complex used in this method to the ferrous form. Therefore, by measuring the formation of Perl's Prussian blue at 700 nm we can monitor the Fe^{2+} concentration; a higher absorbance at 700 nm indicates a higher reducing power.

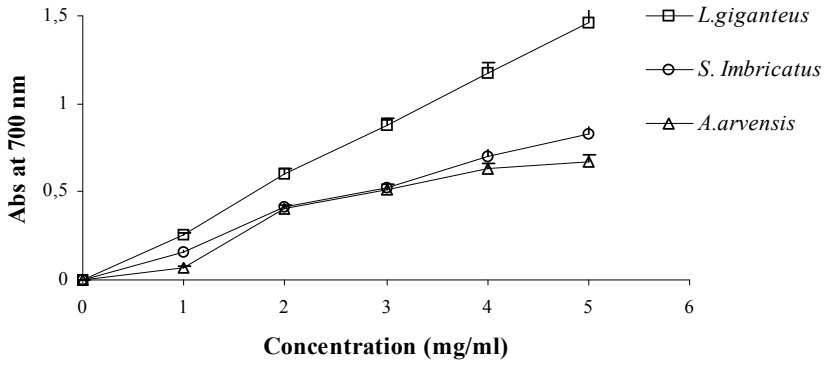


Figure 3.2.1. Reducing power of mushrooms methanolic extracts. Each value is expressed as mean ± standard deviation (n=3).

The reducing power of the mushrooms methanolic extracts increased with concentration. Reducing powers obtained for all the mushrooms were excellent (**Figure 3.2.1** and in the order of *L. giganteus*>*S. imbricatus*~*A. arvensis*. At 5 mg/mL, reducing powers of methanolic extracts from wild edible mushrooms ranged 0.67-1.47, and at 1 mg/mL were 0.072-0.26. Reducing power of BHA at 3.6 mg/mL and α-tocopherol at 8.6 mg/mL were only 0.12 and 0.13, respectively. Methanolic extracts from *A. arvensis* showed only slightly lower reducing power values than *S. imbricatus* (**Figure 3.2.1**). It was reported that the reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Shimada et al., 1992). Accordingly, *L. giganteus* might contain higher amounts of reductone, which could react with free radicals to stabilise and block radical chain reactions.

Radical-scavenging activity (RSA) assay

The free radical DPPH possesses a characteristic absorption at 517 nm (purple in colour), which decreases significantly on exposure to radical scavengers (by providing hydrogen atoms or by electron donation). A lower absorbance at 517 nm indicates a higher radical scavenging activity of the extract. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. This test is a standard assay in antioxidant activity studies and offers a rapid technique for screening the RSA of specific compounds or extracts (Amarowicz et al., 2004).

The RSA values of mushrooms methanolic extracts are presented in **Figure 3.2.2**; results are expressed as the ratio percentage of sample absorbance decrease and the absorbance of DPPH solution in the absence of extract at 517 nm.

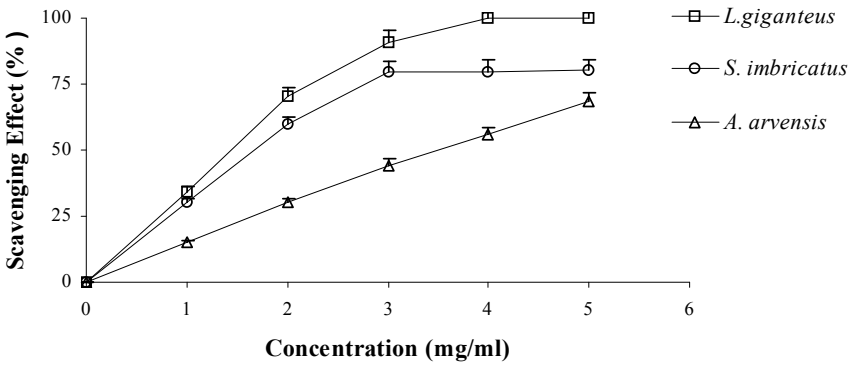


Figure 3.2.2. Scavenging activity (%) on DPPH radicals of mushrooms methanolic extracts. Each value is expressed as mean ± standard deviation (n=3).

From the analysis of figure 3.1.2, we can conclude that the scavenging effects of mushrooms methanolic extracts on DPPH radicals increased with the concentration increase and were excellent for *L. giganteus* (100% at 5 mg/mL), even higher than the scavenging effects of BHA (96% at 3.6 mg/mL) and α-tocopherol (95% at 8.6 mg/mL). The RSA values were good for *S. imbricatus* (80% at 5 mg/mL) and moderate for *A. arvensis* (68.3% at 5 mg/mL).

Assay for erythrocyte hemolysis mediated by peroxyl free radicals

The oxidative hemolysis in erythrocytes induced by AAPH has been extensively studied as model for the peroxidative damage in biomembrane (Zhang et al., 1997). AAPH is a peroxyl radical initiator that generates free radicals by its thermal decomposition and will attack the erythrocytes to induce the chain oxidation of lipid and protein, disturbing the membrane organization and eventually leading to hemolysis. In this study, the protective effect of the mushrooms extracts on hemolysis by peroxyl radical scavenging activity was investigated. **Figure 3.2.3** shows inhibition percentage of hemolysis, as a result of protection against the oxidative damage of cell membranes of erythrocytes from ram, induced by AAPH. The mushroom extracts inhibited hemolysis of erythrocytes in a concentration-dependent manner.

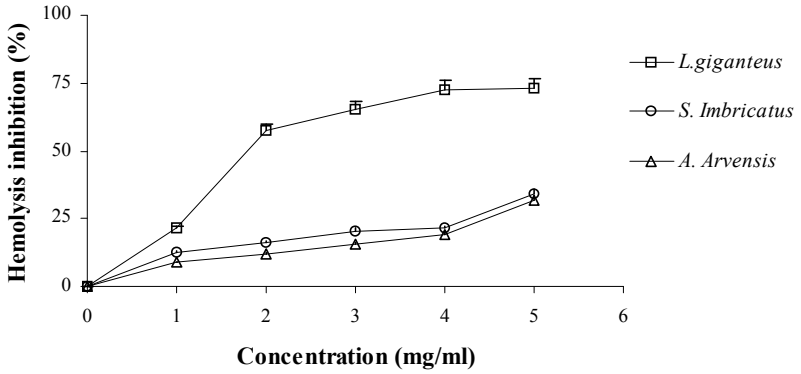


Figure 3.2.3. Hemolysis inhibition (%) of the mushrooms methanolic extracts. Each value is expressed as mean ± standard deviation (n=3).

Once more, *L. giganteus* showed higher protective effect against erythrocytes hemolysis (72.8% at 5 mg/mL) than the other mushrooms (34.2% for *S. imbricatus* and 31.8% for *A. arvensis*). However, the inhibition percentage of the standard L-ascorbic acid on hemolysis of red blood cell was much higher (94.6% at 1 mg/mL) than those of mushroom extracts.

Antioxidant assay using the β-carotene linoleate model system

Figure 3.2.4 shows the antioxidant activity of the mushroom extracts as measured by the bleaching of β-carotene. The antioxidant activity of carotenoid is based on the radical adducts of carotenoid with free radicals from linoleic acid. The linoleic acid free radical attacks the highly unsaturated β-carotene models. The presence of different antioxidants can hinder the extent of β-carotene-bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system (Jayaprakasha et al., 2001).

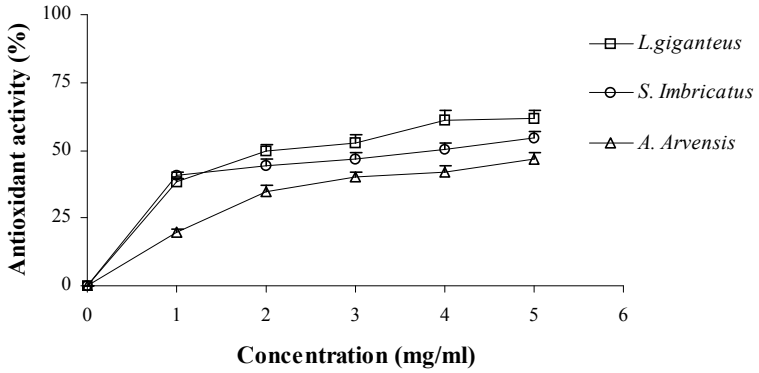


Figure 3.2.4. Antioxidant activity (%) of the mushrooms methanolic extracts by β-carotene bleaching method. Each value is expressed as mean ± standard deviation (n=3).

Accordingly, the absorbance decreased rapidly in samples without antioxidant, whereas in the presence of an antioxidant, they retained their colour, and thus absorbance, for a longer time. Antioxidant activity of *L. giganteus*, *S. imbricatus* and *A. arvensis* extracts increased with their increasing concentration. Their antioxidant activities were 61,4%, 54,3% and 46,7% at 5 mg/mL, but antioxidant activity of TBHQ standard reached 82,2% at 2 mg/mL. It is probable that the antioxidative components in the mushroom extracts can reduce the extent of β-carotene destruction by neutralizing the linoleate free radical and other free radicals formed in the system. Again, *L. giganteus* was the most effective with an EC_{50} value of 2 mg/mL.

In **Table 3.2.2** we present the EC_{50} values for the antioxidant activity assays obtained from each mushroom methanolic extract.

Table 3.2.2. EC_{50} values (mg/mL) of mushroom extracts in the antioxidant activity evaluation assays.

Samples	Reducing power (EC_{50}[a])	DPPH (EC_{50}[b])	Hemolysis inhibition (EC_{50}[c])	β-carotene bleaching (EC_{50}[d])
L. giganteus	1.71	1.44	1.80	2.00
S. imbricatus	2.79	1.67	>5	3.97
A. arvensis	2.86	3.50	>5	>5

[a] EC_{50} (mg/mL): effective concentration at which the absorbance is 0.5.

[b] EC_{50} (mg/mL): effective concentration at which 50% of DPPH radicals are scavenged.

[c] EC_{50} (mg/mL): effective concentration at which 50% of the erythrocytes hemolysis are inhibited.

[d] EC_{50} (mg/mL): effective concentration at which the antioxidant activity is 50%.

Overall, *L. giganteus* revealed better antioxidant properties (lower EC_{50} values) than either *S. imbricatus* or *A. arvensis*, which is in agreement with the higher content of phenol compounds found in the first species. The EC_{50} values obtained for reducing power and scavenging effects on DPPH radicals were better than for hemolysis inhibition mediated by peroxyl free radicals and for the antioxidant activity using the linoleate-βcarotene system. A relationship between the reducing power, DPPH scavenging activity, hemolysis inhibition and β-carotene-bleaching extent was found, supporting that the mechanism of action of the extracts for the antioxidant activity may be identical, being related with the content in total phenols. Though other antioxidants were probably present in these mushroom extracts, the amounts of ascorbic acid, β-carotene and lycopene found in the three Portuguese mushroom extracts were very low, which emphasise the idea that phenolic compounds could make a significant contribution to the mushrooms antioxidant activity. To our best knowledge, the present study was the first report to demonstrate that the antioxidative components in the Portuguese mushrooms extracts can protect the membrane of erythrocytes incubated with AAPH, and reduce the extent of β-carotene destruction by neutralizing the linoleate free radical and other free radicals formed in the system.

3.2.2. Antioxidant activity of Agaricus sp. mushrooms by chemical, biochemical and electrochemical assays

3.2.2.1. Introduction

Living cells, including those of man, animals and plants, are continuously exposed to a variety of challenges that exert oxidative stress. Oxidative stress arises in a biological system after an increased exposure to oxidants, a decrease in the antioxidant capacity of the system, or both. It is often associated with or leads to the generation of reactive oxygen species (ROS), including free radicals, which are strongly implicated in the pathophysiology of diseases, such as cancer, rheumatoid arthritis, cirrhosis and arteriosclerosis, as well as in degenerative processes associated with ageing. Reactive free radicals may come from endogenous sources through normal physiological and metabolic processes such as mitochondrial respiration. Alternatively, they could result from exogenous sources such as exposure to pollutants and ionizing irradiation, and particularly oxygen-derived radicals are capable of oxidizing biomolecules, resulting in cell death and tissue damage (Ames et al., 1993; Chevion et al., 2000; Halliwell and Gutteridge, 1999). Oxidation is also one of the most important processes of food deterioration since it may affect food safety, colour, flavour and texture.

Cells are equipped with several defence systems against free radical damage, including oxidative enzymes such as superoxide dismutase (SOD) and catalase (CAT), or chemical compounds such as α-tocopherol, ascorbic acid, carotenoids, polyphenol compounds and glutathione (Niki et al., 1994). However, antioxidant supplements or antioxidant-containing foods may be used to help the human body to reduce oxidative damage or to protect food quality by preventing oxidative deterioration (Halliwell and Gutteridge, 1999; Elmastas et al., 2007). In recent years, the restriction in the use of synthetic antioxidants, such as BHA (2-*tert*-butyl-4-methoxyphenol) and BHT (2,6-di-tert-butyl-4-methylphenol), has caused an increased interest towards natural antioxidant substances (Branen, 1975, Ames, 1983). Natural antioxidants are being extensively studied for their capacity to protect organisms and cells from damage brought on by oxidative stress, the latter being considered a cause of ageing and degenerative diseases. The antioxidants contained in foods, especially vegetables, are phenolic compounds (phenolic acids and flavonoids), carotenoids, tocopherols and ascorbic acid (Cazzi et al., 1997; Elmastas et al., 2007) that are important protective agents for human health

(Gillman et al., 1995; Block et al., 1998). Mushrooms are rich sources of those compounds (Valentão et al., 2005a, b). In the last years several protocols to determine their antioxidant activity based on spectrophotometric techniques have been reported, although electrochemical techniques have also been tested and developed as an alternative tool for the evaluation of different food extracts, expressed in terms of "antioxidant power", due to their quickness, simplicity and low cost (Chevion et al., 2000; Korotkova et al., 2002; Blasco et al., 2004; Blasco et al., 2005; Cosio et al., 2006).

Antioxidant compounds can act as reduction agents and, in solutions, they tend to be easily oxidized at inert electrodes. Based on this fact, some of the previous cited authors established an interesting relationship between electrochemical behaviour of the antioxidant compounds and their resultant "antioxidant power", where "low oxidation potential" corresponds to "high antioxidant power". On the other hand, ascorbic acid and phenolic compounds are common antioxidants in mushrooms. Electrochemical measurement at positive potentials will then correspond to the oxidation of "total phenolic" and ascorbic acid, plus all compounds with natural antioxidant properties and electrochemical activity, which are present in foods. Blasco et al. (2004) defined an "Electrochemical Index" to express the evaluation of the "total natural antioxidants.

In the present work, cyclic voltammetry and differential pulse voltammetry were used to evaluate *Agaricus sp.* mushrooms antioxidant activity. Moreover, the lipid peroxidation inhibition capacity of the edible mushrooms was assessed by biochemical assays used as models for the lipid peroxidation damage in biomembranes, namelly inhibition of β-carotene bleaching in the presence of linoleic acid radicals, inhibition of erythrocytes hemolysis mediated by peroxyl radicals, and inhibition of thiobarbituric acid reactive substances (TBARS) formation in brain cells. Their antioxidant properties were also evaluated through the reducing power determination and radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. Bioactive compounds such as phenolics, flavonoids, ascorbic acid, β-carotene and lycopene were also determined.

3.2.2.2. Materials and methods

Samples

Five mushrooms species: *Agaricus bisporus* (Lange) Imbach, *Agaricus arvensis* Schaeffer, *Agaricus romagnesii* Wasser, *Agaricus sivaticus* Schaeff., and *Agaricus*

silvicola (Vittadini) Peck were analysed. The first was a commercial sample obtained in a local supermarket. All the others were wild species and were collected in Bragança (Northeast of Portugal), in autumn 2006, under live pine trees (*Pinus* sp.) for the first two species and under oak trees *(Quercus pyrenaica* Willd.) for the last two species. Taxonomic identification was made according to several authors (Moser, 1983; Courtecuisse and Duhem, 1995) and representative voucher specimens were deposited at the herbarium of *Escola Superior Agrária* of *Instituto Politécnico de Bragança*. After collection and taxonomic identification, all the mushrooms were dried in a liophylizator (Ly-8-FM-ULE, Snijders, Holland) before analysis.

Sample preparation

The samples (typically 3 g) were extracted by stirring with 100 mL of methanol at 25 ºC at 150 rpm for 24h and filtered through Whatman nº 4 paper. The residue was then extracted with two additional 100 mL portions of methanol. The combined methanolic extracts were evaporated at 40 ºC to dryness and redissolved in methanol at a concentration of 100 mg/mL, and stored at 4 ºC for further use.

Standards and reagents

Standards BHA (2-*tert*-butyl-4-methoxyphenol), TBHQ (tert-butylhydroquinone), L-ascorbic acid, α-tocopherol, gallic acid and (+)-catechin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin and Ciocalteu's reagent is from Merck (Darmstadt, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Sodium perchlorate monohydrate, puriss grade, was purchased from Fluka (St. Gallen, Switzerland), and kept at 30ºC before use. All other chemicals were obtained from Sigma. Methanol was obtained from Pronalab (Lisbon, Portugal). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

Chemical assays

Determination of bioactive components. Bioactive compounds in the mushrooms extracts were determined by colorimetric assays, based on procedures described in chapter **3.1.4.2.** (*Total bioactive compounds*).

DPPH radical-scavenging activity and *reducing power* were evaluated as described in chapter **3.2.1.2**.

Biochemical assays

Inhibition of β-carotene bleaching and *inhibition of erytrocyte hemolysis mediated by peroxyl free radicals* were evaluated as described in chapter **3.2.1.2**.

Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS). Brains were obtained from pig (*Sus scrofa*) of body weight ~150 Kg, dissected and homogenized with a Polytron in ice-cold Tris–HCl buffer (pH 7.4, 20 mM) to produce a 1:2 (w/v) brain tissue homogenate which was centrifuged at 3,000*g* for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with the mushrooms extracts (0.2 mL) in the presence of $FeSO_4$ (10 μM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, 0.38 mL), and the mixture was then heated at 80 ºC for 20 min. After centrifugation at 3,000*g* for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at λ=532 nm (Ng et al., 2000). The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A – B)/A] x 100%, where A and B were the absorbance of the control and the compound solution, respectively. The extract concentration providing 50% lipid peroxidation inhibition (EC_{50}) was calculated from the graph of antioxidant activity percentage against extract concentration. BHA was used as standard.

Electrochemical characterisation

Instrumentation. Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements were performed on an Autolab PGSTAT 302 potentiostat/galvanostat using a closed standard three-electrode cell. A glassy carbon (BAS, $\phi = 0.314$ cm^2) was use as the working electrode and a Pt foil as the counter electrode. All potentials are referred to an Ag/AgCl 3 M KCl reference electrode (Methrom, Courtaboeuf Cedex, France). Prior to use, the working electrode was polished in an aqueous suspension of 0,3 μm alumina (Buehler, Illinois, USA) on a Master-Tex (Buehler) polishing pad, then rinsed with water. Subsequently, in a chemical treatment, the electrode was sonicated in HCl 6 M during 1 min and then in methanol. This cleaning procedure was applied always before any electrochemical measurements.

Procedure. All the mushroom extracts and standard compounds were studied in methanol/acetate buffer 0.1 M (pH 4) / $NaClO_4$ (70:28:2) solutions. For calibration standards (ascorbic and gallic acids), the concentration was set between 0.1 and 1 mM, while for mushroom extracts solutions the concentration was changed between 0.5 and 15 mg/mL. All solutions were analysed immediately after preparation and the electrochemical responses recorded after the glassy carbon electrode immersion, to minimize adsorption of species onto the electrode surface prior to run.

Cyclic voltammetry was used to characterize the electrochemical responses between 0 and 1.2 V, at 0.1 Vs^{-1}, whereas the antioxidant power was evaluated by DPV; operating conditions for DPV were set at 60 mV pulse amplitude and 0.030 Vs^{-1} as scan rate. For each extract, the current density was plotted as a function of mushroom extract mass and compared with those of the standards.

Statistical analysis

For each one of the *Agaricus* sp., three samples were analysed and also all the assays were carried out in triplicate. The results are expressed as mean values and standard error (SE) or standard deviation (SD). The mushrooms antioxidant activity was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SAS v. 9.1.3 program.

3.2.2.3. Results and discussion

Evaluation of antioxidant properties by chemical and biochemical assays

Several chemical and biochemical assays were used to screen the antioxidant properties: reducing power, scavenging activity on DPPH radicals, inhibition of β-carotene bleaching, and inhibition of lipid peroxidation in brain tissue. The assays were performed in the whole mushroom extract, since it could be more beneficial than isolated constituents; a bioactive individual component can change its properties in the presence of other compounds present in the extracts. According to Liu (2003) additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent bioactive properties and the benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals present in whole foods. This would explain why no single antioxidant can replace the combination of natural phytochemicals to achieve the health benefits.

Table 3.2.3 shows EC_{50} values obtained in the antioxidant activity assays of *Agaricus* sp. mushrooms. All the species proved to have antioxidant activity, namely radical scavenging activity and lipid peroxidation inhibition capacity. The antioxidant activity was better (lower EC_{50} values) in chemical assays than in the biochemical assay using animal cells.

Table 3.2.3. EC_{50} values (Mean ± SD) obtained in the antioxidant activity assays of different *Agaricus* species. In each column different letters mean significant differences ($p<0.05$).

	Antioxidant properties (EC_{50} values; mg/mL)				
	DPPH scavenging activity	Reducing power	β-carotene bleaching inhibition	Hemolysis inhibition	TBARS inhibition
A. arvensis	15. 85 ± 0.27 a	4.20 ± 0.04 a	48.30 ± 0.83 a	> 50 a	> 50 a
A. bisporus	9.61 ± 0.07 b	3.63 ± 0.02 b	21.39 ± 0.45 b	> 50 a	46.82 ± 0.03 b
A. silvícola	6.39 ± 0.16 c	3.24 ± 0.01 c	14.75 ± 1.57 c	43.75 ± 0.11 b	31.97 ± 0.04 c
A. silvaticus	5.37 ± 0.06 d	2.08 ± 0.05 e	3.72 ± 0.21 d	22.15 ± 0.31 d	17.79 ± 0.10 e
A. romagnesii	6.22 ± 0.10 c	2.23 ± 0.01 d	4.36 ± 0.86 d	35.38 ± 0.9 c	22.38 ± 0.05 d

Table 3.2.4 presents phenolic, flavonoids, ascorbic acid and carotenoids concentrations obtained in the five *Agaricus* sp. mushroom extracts. Phenolics (2.72-8.95 mg/g) were the major antioxidant components found in the extracts, followed by flavonoids (1.65-3.88 mg/g). Ascorbic acid was found in small amounts (0.02–0.04 mg/g), and β-carotene and lycopene were only found in vestigial amounts (<9 μg/g), which is in agreement with previous studies in other mushroom species (see chapter **3.2.1**). *Agaricus silvaticus* revealed a higher content in phenolic and flavonoids compounds, which significantly ($p<0.05$) decreased in the other species.

Table 3.2.4 Bioactive compounds (Mean ± SD) obtained for *Agaricus* sp. In each column different letters mean significant differences ($p<0.05$).

Sample	Total phenolics (mg/g)	Flavonoids (mg/g)	Ascorbic acid (mg/g)	β-carotene (μg/g)	Lycopene (μg/g)
A. arvensis	2.72 ± 0.17 d	1.65 ± 0.09 d	0.02 ± 0.00 c	8.52 ± 0.38 a	4.70 ± 0.25 a
A. bisporus	4.49 ± 0.16 c	1.73 ± 0.11 d	0.03 ± 0.01 b	1.95 ± 0.10 d	0.91 ± 0.06 d
A. romignesii	6.18 ± 0.44 b	2.87 ± 0.12 c	0.04 ± 0.00 a	1.32 ± 0.03 e	0.38 ± 0.02 e
A. silvaticus	8.95 ± 0.30 a	3.88 ± 0.04 a	0.04 ± 0.00 a	5.42 ± 0.10 b	2.63 ± 0.06 b
A. silvícola	6.40 ± 0.17 b	3.40 ± 0.01 b	0.04 ± 0.00 a	3.02 ± 0.12 c	1.14 ± 0.08 c

In fact, *A. silvaticus* was the most efficient species (lower EC_{50} values) concerning antioxidant activity, while *A. arvensis* presented lower antioxidant properties (higher EC_{50} values), which are compatible to its lower phenols content. Some authors have already reported a direct correlation between mushrooms antioxidant activity and total phenolic content, although the antioxidant action is raised by other substances such as tocopherols and β-carotene (Cheung et al., 2003). The bioactivity of phenolics may be related to their ability to chelate metals, inhibit lipoxygenase and scavenge free radicals (Decker, 1997). Also, in food systems, flavonoids can act as free radical scavengers and terminate the radical chain reactions that occur during the oxidation of triglycerides. Therefore, they present antioxidative efficiency in oils, fats and emulsions (Roedig-Penman and Gordon, 1998).

Evaluation of antioxidant properties by electrochemical techniques

Figure 3.2.5 a and **b** shows the cyclic voltamogram of the standards ascorbic and gallic acids, respectively. Both compounds present typical irreversible oxidation processes, as observed in many antioxidant substances (Gunckel et al., 1998; Kilmartin et al., 2001; Cosio et al., 2006), with one anodic peak at E_{pa}=0.26 V for ascorbic and two anodic peaks at $E(I)_{pa}$=0.36 V, $E(II)_{pa}$=0.62 V for gallic acid. The same irreversible electrochemical behaviour was observed for all *Agaricus sp.* extracts (**Figure 3.2.5 c**), although with oxidation potentials more positive than the standards, around 0.9 V. These results showed that both standards are oxidised at less positive potentials than the compounds responsible for the electrochemical responses of the mushrooms extracts, and indicated that, under the electrochemical conditions used, neither of the standards were present in the mushroom extracts.

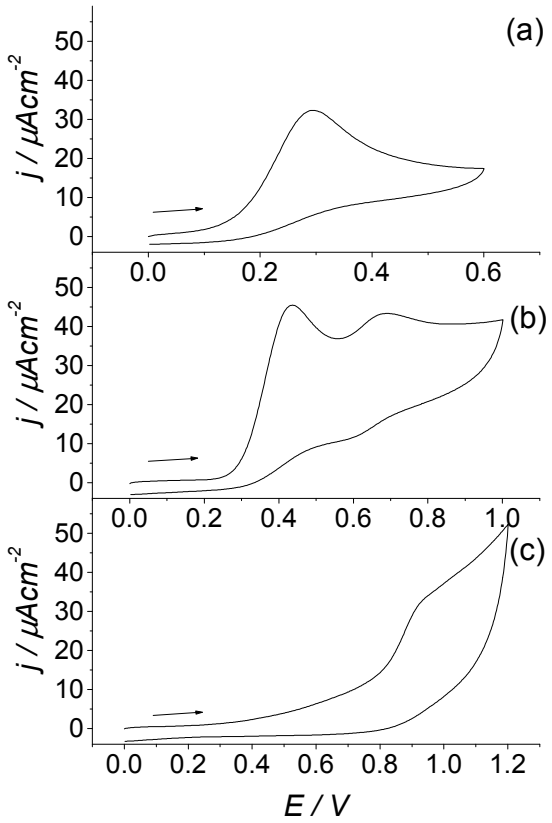


Figure 3.2.5. Cyclic voltammogram, at 0.1 Vs^{-1}, of methanol/ acetate buffer 0.1 M (pH 4)/ $NaClO_4$ (70:28:2) solutions of: (a) 0.5 mM ascorbic acid, (b) 0.5 mM gallic acid, (c) 5 mg/mL extract of *A. silvaticus*.

The similarity in the oxidation potential between all the *Agaricus* species studied (**Table 3.2.5**) indicates that the different extracts should have an analogous composition in respect to the electroactive species.

Table 3.2.5. Electrochemical results from cyclic voltammetry and differential pulse voltammetry of the methanolic mushroom extracts.

Sample	$E_{1/2}$ / V	Slope / $\mu Acm^{-2}\ mg^{-1}mL$	A. P. (AA) mg/g	A. P. (GA) mg/g
A. arvensis	0.90	0.55	3.1	2.7
A. bisporus	0.92	0.79	1.9	1.6
A. romagnesii	0.92	0.50	2.8	2.4
A. silvaticus	0.92	0.85	4.4	3.7
A. silvicola	0.94	0.85	4.6	4.0
Ascorbic acid	0.26	204.75	1000	616
Gallic acid	0.36/0.62	247.81	1302	1000

A.P.- antioxidant power; AA- ascorbic acid; GA- gallic acid

Cyclic voltammetry is frequently used for the characterization of electroactive systems. However, when organic substances are present, there are great chances of adsorption phenomena on the electrode surface, limiting the use of this technique to quantitative measurements. To overcome this constriction and gain quantitative information in the antioxidant capabilities of the extracts, the differential pulse voltammetry was used. In this technique the current is measured before and after the potential pulse application allowing the discrimination of effects, like absorption on the electrode, that are approximately constants in a certain potential interval (Brett and Brett, 1993).

Figure 3.2.6 shows the differential pulse voltamograms for the mushroom extracts. They resemble the behaviour of the cyclic voltamogram responses, with one peak at the same potential values. The exception is *A. bisporus* extract where it is possible to observe a broad peak around 0.6 V. In fact, with this technique the peaks are better resolved than in CV, overcoming the difficulties in accessing a correct baseline.

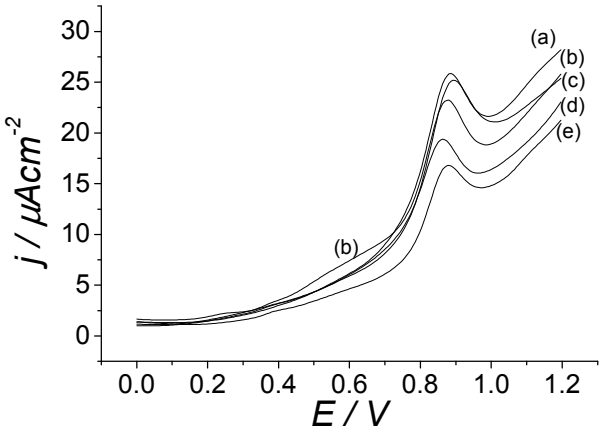


Figure 3.2.6. Differential pulse voltammograms of 10 mg/mL *Agaricus* methanolic extracts in methanol/ acetate buffer 0.1 M (pH 4)/ $NaClO_4$ (70:28:2) solutions. (a) *A. silvicola*, (b) *A. bisporus*, (c) *A. silvaticus*, (d) *A. arvensis*, (e) *A. romagnesii*.

The differential pulse voltammograms, at several concentrations of gallic acid, are shown in **Figure 3.2.7**. As can be seen there is an increase in peak current with the increase in gallic acid concentration, which lead to a linear relation between the two parameters.

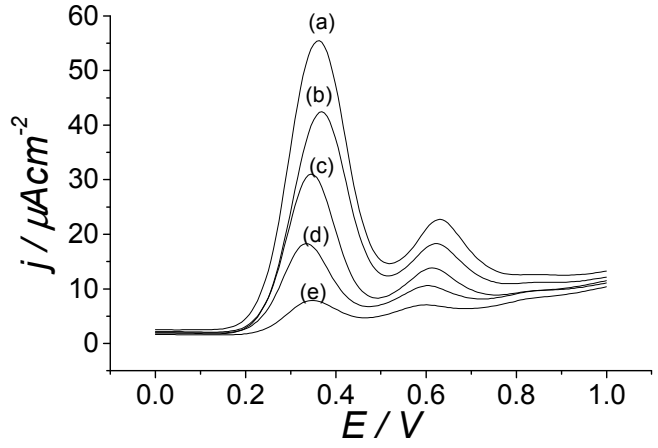


Figure 3.2.7. Differential pulse voltammograms of gallic acid in methanol/ acetate buffer 0.1 M (pH 4)/ NaClO4 (70:28:2) solutions. (a) 0.20 mg/mL, (b) 0.15 mg/mL, (c) 0.1 mg/mL, (d) 0.05 mg/mL, (e) 0.02 mg/mL.

The same behaviour was found for ascorbic acid and mushroom extracts (**Figure 3.2.8 and Table 3.2.5**) although the slopes for the plots peak current density *vs* extract concentration are very different. For this technique the peak current density, *j*, depends, not only on the concentration, but also on the electron transfer kinetics and the diffusion coefficient of the electroactive species (or the average of several species) (Brett and Brett, 1993), preventing the direct comparison between the standards and samples data. Moreover, the standards tested were not detected within the mushrooms extracts, at an electrochemically detectable concentration, and therefore the relation between current density and concentration for the mushroom extracts will differs from that of gallic and ascorbic acid.

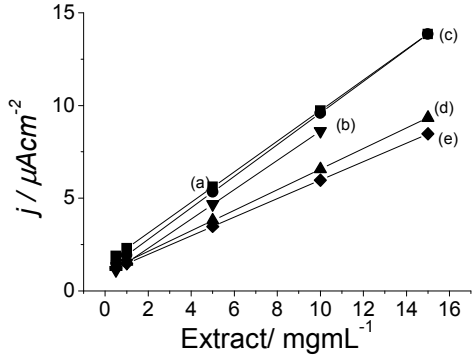


Figure 3.2.8. Variation of the peak current density, in DPV voltamograms, with extract concentration. (a) *A. silvicola*, (b) *A. bisporus*, (c) *A. silvaticus*, (d) *A. arvensis*, (e) *A. romagnesii*.

The difference between the slopes of the two standards in **Table 3.2.5** reflects the difference between their diffusion coefficients. However, when compared with the extracts slopes, the enormous discrepancy cannot be explained on the basis of their different diffusion coefficients (species belong to a chemically comparable family of phenolic compounds), but to the amount of effective electroactive mass in the mushroom extract composition. Based on the similarity in the cyclic voltammograms of *Agaricus sp,* it can be assumed that the species responsible for the electrochemical activity are chemically similar as well as their diffusion coefficient. In this context and based on the slopes values of peak current density *vs* extract concentration plots, it can be concluded that the amount of electroactive phenolic compounds in *A. bisporus, A. silvaticus* and *A. silvicola* are almost double of that present in the extract of *A. arvensis* and *A. romagnesii.*

In order to express the "antioxidant power" of the mushrooms extracts in equivalent terms the results were compared with those of the standards. It must be mentioned that at very low and high concentrations of extract there are significant deviations in linearity of *j vs mass*, most probably due to adsorption phenomena on the electrode. The values are presented in **Table 3.2.5**, and are expressed in terms of either gallic and ascorbic acid. These results show that *A. silvicola* and *A. silvaticus* exhibit the highest "antioxidant power" in agreement with the results obtained in the biochemical assays. The values obtained from the electrochemical experiment are lower than those resulting from Folin Ciocalteu´s assay. This outcome is frequently observed when comparing the colorimetric method with others and is attributed to the overestimation of the "total polyphenolic" content due to the interferences of other non-phenolic species like reducing sugars (Blasco et al., 2005).

Overall, all the species proved to have antioxidant properties, namely radical scavenging activity and lipid peroxidation inhibition capacity. By using electrochemical techniques, it was also proved that mushroom extracts have a similar composition on the electroactive species, which exhibit oxidation potentials more positive than the standards. *A. silvaticus* was the most efficient species presenting the lowest EC_{50} values in the chemical and biochemical assays, and the highest "antioxidant power" in the electrochemical assays. Finally, the work described in this study shows that cyclic voltammetry and differential pulse voltammetry can be considered as important techniques for the evaluation of mushrooms antioxidant properties.

3.2.3 Antimicrobial activity and bioactive compounds of Portuguese wild edible mushrooms methanolic extracts

3.2.3.1. Introduction

Mushrooms are a nutritionally functional food and a source of physiologically beneficial medicines (Sagakami et al., 1991; Wasser and Weis, 1999a). Both fruiting body and the mycelium contain compounds with wide-ranging antimicrobial activity. Mushrooms are rich sources of natural antibiotics; in these, the cell wall glucans are well-known for their immunomodulatory properties, and many of the externalized secondary metabolites (extracellular secretions by the mycelium) combat bacteria (Benedict and Brady, 1972; Krupa et al., 1979) and viruses (Suzuki et al., 1990; Collins and Ng, 1997; Eo et al., 1999; Brandt and Piraino, 2000). Additionally, the exudates from mushroom mycelia are active against protozoa such as the parasite that causes malaria, *Plasmodium falciparum* (Lovy et al., 1999; Isaka et al., 2001). The importance of the Chinese Shiitake mushroom (*Lentinus edodes*) is well-known; besides its anti-tumour activity, it has been demonstrated to increase the host resistance to bacterial and viral infections (Jong and Birningham, 1993). Several compounds extracted from this mushroom revealed antifungal and antibacterial activity (Morita and Kobayashi, 1967; Yasumoto et al., 1971), namely against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* (Takazawa et al., 1982). The chloroform and ethylacetate extracts of the dried mushroom have antibacterial activity against *Streptococcus mutans* and *Prevotella intermedia* (Hirasawa et al., 1999). A few studies have been reported on the antimicrobial activity of other edible mushrooms and its bioactive compounds. *Laetiporus sulphurous* antioxidant and antimicrobial activities were reported and correlated to the phenols and flavonoids contents (Turkoglu, et al., 2007), and guaiane sesquiterpenoids isolated from the fruit bodies of edible *lactarius* species proved to have antibacterial activity (Anke et al., 1989).

The Northeast of Portugal is one of the European regions with higher wild edible mushrooms diversity, some of them with great gastronomic relevance. The aim of this work was to investigate the antimicrobial properties of phenolic-rich extracts of different Portuguese wild edible mushrooms. Phenolic compounds have attracted much interest because *in vitro* and *in vivo* studies suggest that they have a variety of beneficial biological properties, which may play an important role in the maintenance of human

health. Phenolics are antioxidants with redox properties, which allow them to act as reducing agents, hydrogen donators, free radical scavengers (Ferreira et al., 2007), and singlet oxygen quenchers (Pietta, 2000). They also have metal chelation properties and inhibit lipid peroxidation (Rice-Evans et al., 1997; Kähkönen, 1999) Their significance for the human diet and antimicrobial activity has been recently established (Rauha et al., 2000; Nychas et al., 2003). Some phenolic compounds exhibit various physiological activities including anti-inflammatory, antiallergic, anticarcinogenic, antihypertensive, antiarthritic and antimicrobial activities (Vaquero et al., 2007).

In fact, the enhanced prevalence of infectious diseases is becoming a world-wide problem, and the resistance problem demands renewed efforts to be made to seek antimicrobial agents effective against pathogenic microorganisms resistant to current treatments. Therefore, the search for new products with antimicrobial properties is a very active domain of research, and herein we suggest natural resources for that purpose.

We report a screening of antibacterial activities using Gram positive (*Bacillus cereus*, *B. subtilis*) and Gram negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*) and antifungal (*Candida albicans*, *Cryptococcus neoformans*) performed for three wild edible mushroom species, *Lactarius deliciosus* (L.) Gray, *Sarcodon imbricatu*s (L.) P. Karst., and *Tricholoma portentosum* (Fr.) Quél. from Northeast of Portugal. Extracts from the entire mushroom, the cap and the stipe individually were compared for their activities and bioactive compounds present in each portion.

3.2.3.2. Materials and methods

Microorganisms and culture conditions

The bacterial strains used as test organisms were *Bacillus cereus* CECT 148, *B. subtilis* CECT 498, *Escherichia coli* CECT 101, and *Pseudomonas aeruginosa* CECT 108, obtained from the Spanish type culture collection (CECT) of Valencia University. The fungi strains used were *Candida albicans* CECT 1394 from the same collection and *Cryptococcus neoformans* ESA 1, obtained in the Microbiology Laboratory of *Escola Superior Agrária de Bragança*. Microorganisms were cultured aerobically at 37 ºC in nutrient agar medium for bacteria, and at 30 ºC in sabouraud dextrose agar medium for fungi (Scientific 222 model). Before experimental use, cultures from solid medium were subcultivated in liquid media, incubated (Stuart Scientific SI50 model) and used as the

source of inoculums for each experiment.

Standards and Reagents

Gallic acid, (+)-catechin and Folin and Ciocalteu's reagent were from Merck (Darmstadt, Germany). Methanol was obtained from Pronalab (Lisboa, Portugal). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, USA). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

Samples

Samples of *Lactarius deliciosus* (L.) Gray, *Sarcodon imbricatu*s (L.) P. Karst., and *Tricholoma portentosum* (Fr.) Quél. were collected under live pine trees (*Pinus* sp.), in Bragança (Northeast of Portugal), in autumn 2004. After collection, the mushrooms were grouped by taxon and were air-dried in a liophylizator (Ly-8-FM-ULE, Snijders) before analysis. Taxonomic identification was made according to several authors (Marchand, 1971-1986; Moser, 1983; Bon, 1988; Courtecuisse and Duhem, 1995; Courtecuisse, 1999) and representative voucher specimens were deposited at the herbarium of *Escola Superior Agrária* of *Instituto Politécnico de Bragança*.

Sample preparation

A fine dried mushroom powder (20 mesh) sample (10 g) was extracted by stirring with 100 mL of methanol at 25 ºC at 150 rpm for 24h and filtered through Whatman nº 4 paper. The residue was then extracted with two additional 100 mL portions of methanol, as described earlier. The combined methanolic extracts were evaporated at 40 ºC to dryness.

For agar diffusion assays, the phenolic compounds were dissolved in DMSO and filter-sterilized through a 0.22 μm membrane filter. The range of phenolic compounds concentrations used included 500 mg/mL to 0.1 mg/mL. All the assays were performed using the entire mushroom, the cap or the stipe separately.

Determination of bioactive components

Phenolic compounds, flavonoids, ascorbic acid, β-carotene and lycopene were determined according to the procedures described in **3.1.4.2** (*Total bioactive compounds*).

Antimicrobial activity

A screening of antibacterial activities with two Gram-negative (*E. coli*, *P. aeruginosa*) and two Gram-positive bacteria (*B. subtilis*, *B. cereus*) was performed; antifungal activity (*C. albicans, C. neoformans*) was also assessed according to the procedure described in **3.1.4.2** (*Test assays for antimicrobial activity*).

3.2.3.3. Results and discussion

Three wild edible mushroom species (*L. deliciosus*, *S. imbricatus*, *T. portentosum*) from the Northeast of Portugal were evaluated for their content in total phenols, flavonoids ascorbic acid, β-carotene and lycopene, and the obtained extracts were screened for their antimicrobial activity. The assays were carried out using the DMSO solutions of the entire mushroom, the cap and the stipe separately. DMSO was chosen as solvent after comparative toxicity assays (data not shown) and was not toxic.

Table 3.2.6 shows the concentrations of the bioactive compounds found in the mushroom extracts. Whereas total phenols and flavonoids were the major components found in the mushroom extracts, ascorbic acid was found in small amounts (0.18-0.52 mg/g), and β-carotene and lycopene were only found in vestigial amounts (< 91 μg/g).

Table 3.2.6. Contents[a] of bioactive compounds found in the mushroom extracts.

Mushroom sample		Total phenols (mg/g)	Flavonoids (mg/g)	Ascorbic acid (mg/g)	β-carotene (μg/g)	Lycopene (μg/g)
L. deliciosus	Total	17.25 ± 0.65	8.14±0.81	0.24 ±0.02	90.10 ±4.76	40.71 ±3.45
	Cap	10.66 ± 0.52	4.76±0.11	0.21 ±0.01	51.00 ±3.12	25.83 ±2.12
	Stipe	6.31 ± 0.29	3.59±0.16	0.18 ±0.01	18.50 ±1.25	5.69 ±0.62
S. imbricatus	Total	3.76 ± 0.11	2.82±0.09	0.50 ±0.04	20.40 ±1.33	10.51 ±1.00
	Cap	2.59 ± 0.10	1.72±0.08	0.36 ±0.02	12.54 ±1.11	6.03 ±0.63
	Stipe	1.50 ± 0.06	1.46±0.08	0.19 ±0.01	7.84 ±0.75	4.48 ±0.60
T. portentosum	Total	10.80 ± 0.47	0.40±0.02	0.52 ±0.04	-	-
	Cap	6.57 ± 0.31	-	0.31±0.02	-	-
	Stipe	3.91 ± 0.17	-	0.22±0.02	-	-

[a] Each value is expressed as mean ± standard deviation (n=3); (-) Not detected.

The portion of the mushroom used (entire, cap or stipe) had influence in the results obtained. The extracts with the entire mushroom showed higher phenolic and flavonoid

contents either than the cap or the stipe. Also the amount of phenolic and flavonoid compounds in the cap methanolic extracts was higher than the amount found in stipe extracts.

Table 3.2.7 shows the antimicrobial screening of phenolic compounds extracted from *L. deliciosus*, *S. imbricatus and T. portentosum* against *B. cereus*, *B. subtilis* (Gram +), *E. coli*, *P. aeruginosa* (Gram -) bacteria, and *C. albicans* and *C. neoformans* (fungi). The MICs for bacteria and fungi were determined as an evaluation of the antimicrobial activity of the tested mushrooms. The diameters of the inhibition zones corresponding to the MICs are also presented.

Table 3.2.7. Antimicrobial activity of the mushroom extracts.

	MIC (mg/mL)					
Mushroom	*B. cereus*	*B. subtilis*	*E. coli*	*P. aeruginosa*	*C. albicans*	*C. neoformans*
L. deliciosus (total)	10 (+ + + +)	100 (+ + + +)	300 (-)	100 (+ + + +)	50 (+ + + +)	10 (+ + + +)
L. deliciosus (cap)	10 (+ + +)	300 (+ + + +)	300 (-)	300 (+ + + +)	100 (+ + + +)	10 (+ + +)
L. deliciosus (stipe)	50 (+ + + +)	300 (-)	300 (-)	300 (+ + +)	300 (-)	50 (+ + + +)
S. imbricatus (total)	10 (+ + +)	300 (-)	300 (-)	300 (-)	300 (-)	300 (+ + + +)
S. imbricatus (cap)	100 (+ + + +)	300 (-)	300 (-)	300 (-)	300 (-)	300 (-)
S. imbricatus (stipe)	300 (+)	300 (-)	300 (-)	300 (-)	300 (-)	300 (-)
T. portentosum (total)	100 (+ + + +)	300 (+ + + +)	300 (-)	300 (-)	300 (-)	300 (-)
T. portentosum (cap)	100 (+ + +)	300 (+ + +)	300 (-)	300 (-)	300 (-)	300 (+ + + +)
T. portentosum (stipe)	300 (+ + + +)	300 (-)	300 (-)	300 (-)	300 (-)	300 (-)
Ampicillin	0.00313 (+ + + +)	0.0125 (+ + + +)	0.00625 (+ + + +)	0.00625 (+ + + +)	NT	NT
Cycloheximide	NT	NT	NT	NT	0.0125 (+ +)	0.00625 (+ + + +)

No antimicrobial activity (-), inhibition zone < 1 mm. Slight antimicrobial activity (+), inhibition zone 2-3 mm. Moderate antimicrobial activity (+ +), inhibition zone 4-5 mm. High antimicrobial activity (+ + +), inhibition zone 6-9 mm. Strong antimicrobial activity (+ + + +), inhibition zone > 9 mm. Standard deviation ± 0.5 mm. NT- Not tested.

All the mushrooms revealed antimicrobial activity showing different selectivity and MICs for each microorganism (**Figure 3.2.9**). *L. deliciosus* showed better results than *T. portentosum* and *S. imbricatus* (lower MICs), which is in agreement with the higher content of phenols and flavonoids found in the first species (**Table 3.2.7**). The entire and the cap mushroom extracts of *L. deliciosus* inhibited *B. cereus*, *B. subtilis*, *P. aeruginosa*, *C. albicans* and *C. neoformans*, while the stipe mushroom extract only inhibited *B. cereus*, *P. aeruginosa* and *C. neoformans*. This mushroom showed lower MICs for the Gram + bacteria and it was the only that revealed activity against *P. aeruginosa* (Gram – bacteria) and *C. albicans* (fungi).

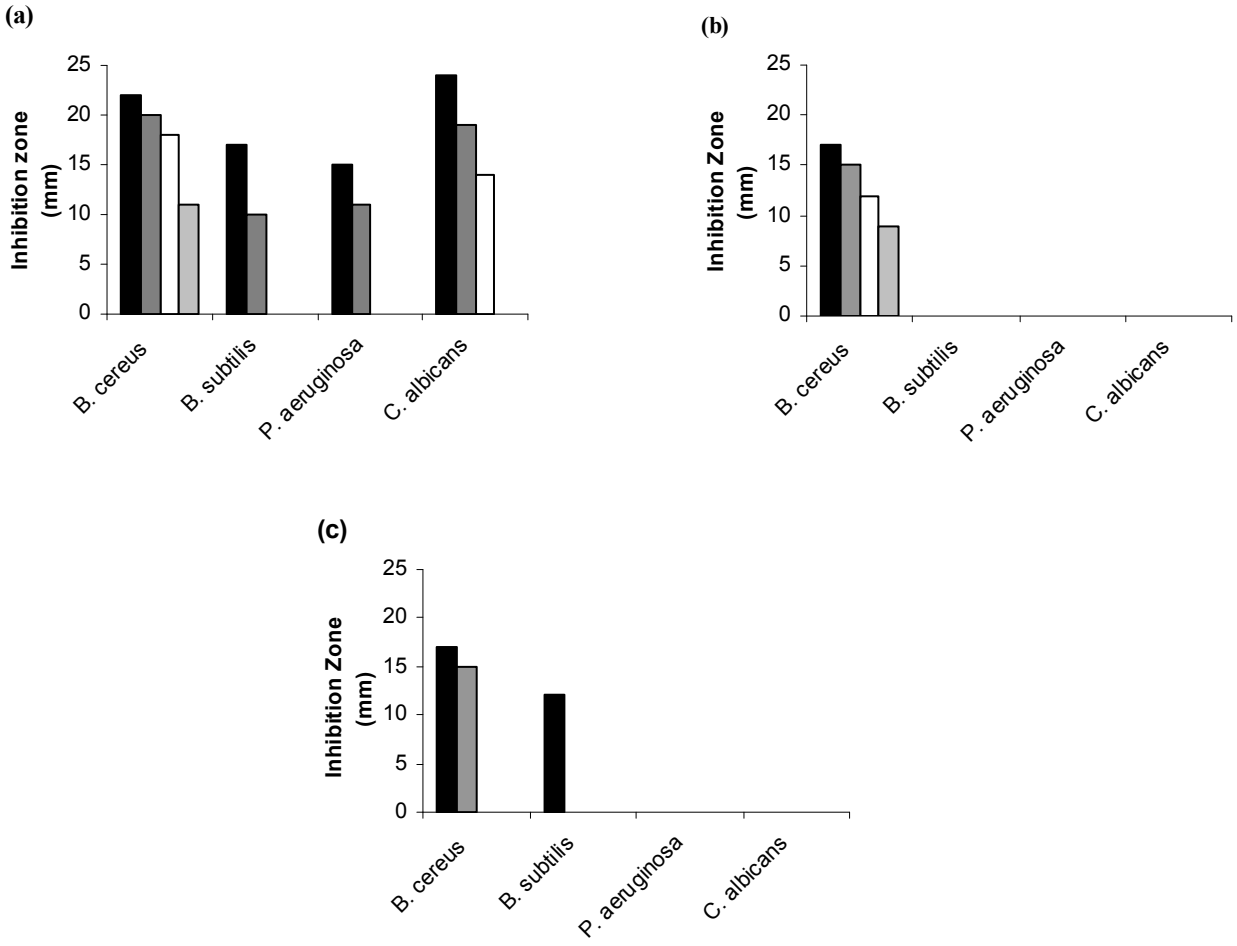


Figure 3.2.9. Antimicrobial activity of the extracts of different entire mushrooms against selected microorganisms. (a) L. deliciosus, (b) S. imbricatus and (c) T. portentosum. (■) concentration of 300 mg/mL, (■) concentration of 100 mg/mL, (□) concentration of 50 mg/mL and (□) concentration of 10 mg/mL.

The *T. portentosum* extract was effective only against Gram + bacteria (*B. cereus*, *B. subtilis*) and *C. neoformans*. When the cap was used the same selectivity was obtained,

but with higher MICs; the stipe extract was only effective against *B. cereus*. In fact, the contents in total phenols and flavonoids for the stipe extracts were always lower than in the other extracts. As expected due to its lower content in bioactive compounds, *S. imbricatus* was the less effective (higher MICs) mushroom, showing activity only against *B. cereus* and *C. neoformans*. The cap extract was selective for *B. cereus*, while the stipe extract was not effective against the tested microorganisms.

E. coli was resistant to all the mushrooms extracts. As expected, the standards ampicillin (antibacterial) and cycloheximide (antifungal) presented lower MICs than the mushrooms extracts. Usually, pure active compounds reveal more activity than crude extracts. Searching wild sources may bring new natural products with antimicrobial properties that provide good protection against the infectious diseases. Therefore, new wild edible mushrooms, as natural sources, could be introduced for this purpose.

The above findings point out that the antimicrobial activity is correlated with the content in total phenols and flavonoids. The use of the entire mushroom is recommended and the mushroom cap proved to be better than the stipe. The extracts of *L. deliciosus*, *S. imbricatus* and *T. portentosum* inhibited some medically important microorganisms. This suggests that they are potential sources of new antimicrobial agents. With an increasing number of bacteria developing resistance to commercial antibiotics, such as MSRA (methicillin-resistant *S. aureus* and *Pseudomonas*), extracts and derivatives from mushrooms hold great promise for novel medicines in modern times. As far as our literature survey could ascertain, a few information was available on the *in vitro* antimicrobial activities of European wild mushrooms and it is the first time that Portuguese wild edible mushrooms (entire, cap and stipe) were submitted to these studies.

3.2.4 Chemical composition and biological properties of Portuguese wild mushrooms: A comprehensive study

3.2.4.1. Introduction

In recent years oxidative stress, induced by reactive oxygen species (ROS) that are generated by normal metabolic activity as well as lifestyle factors such as smoking, exercise and diet, has been implicated in the causation and progression of several chronic diseases. Antioxidants that can mitigate the damaging effects of ROS have been the focus of recent research (Halliwell, 1996). There is convincing scientific evidence in support of the association between diet and chronic diseases such as cancer, cardiovascular disease, diabetes and osteoporosis. Epidemiological studies have consistently shown an inverse association between consumption of vegetables and fruits and the risk of certain forms of cancer and cardiovascular diseases (Bazzano et al., 2001). Although the protective effects have been primarily attributed to well-known antioxidants, such as vitamin C, vitamin E and β-carotene, plant phenolics may also play a significant role (Soorattee et al., 2005).

Phenolic compounds exhibit a wide range of biological effects including antibacterial, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic and vasodilatory actions (Soorattee et al., 2005); many of these biological functions have been attributed to their free radical scavenging and antioxidant activity. Flavonoids are the most common and widely distributed group of plant phenolics, and have been shown to be highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and various free radicals, which are possibly involved in DNA damage and tumor promotion (Marchand, 2002). The health benefits of tocopherol as a bioactive compound are well documented. α-Tocopherol, the principal form of vitamin E, is a lipid-soluble antioxidant and it functions as a chain-breaking antioxidant for lipid peroxidation (LP) in cell membranes and also as a scavenger of ROS such as singlet oxygen. It is considered to serve as the first line of defence against LP, and it protects PUFAs (polyunsaturated fatty acids) in cell membranes from free radical attack through its scavenging activity in biomembranes at early stages of LP (Kanu et al., 2007). The antioxidant properties of carotenoids have been suggested as being the main responsible for their beneficial effects (Rao and Rao, 2007). Particularly, β-carotene has been found to be inversely associated with cancer

risk in epidemiologic studies and showed promising results in laboratory assays. Also, the role of lycopene in the prevention of chronic diseases has been evaluated in epidemiological studies as well as in tissue culture experiments using human cancer cell lines, animal studies and also human clinical trials (Rao and Rao, 2007).

The biological properties of mushrooms, namely antioxidant (Mau et al., 2002b; Lo and Cheung, 2005) and antimicrobial properties (Hirasawa et al,1999; Hatvani et al., 2001; Turkoglu et al., 2007), have been described and attributed to their high content in antioxidants (Valentão et al., 2005; Elmastas et al., 2007; Tsai et al., 2007) and antibiotics, respectively. Besides their pharmacological characteristics, wild mushrooms are becoming more and more important in our diet for their nutritional value, including relatively high protein and low fat/energy contents (Longvah and Deosthale, 1998, Díez and Alvarez, 2001; Agahar-Murugkar, 2005). The fatty acid composition may also have beneficial effects on blood lipid profiles. Substitution of SFAs (saturated fatty acids) with MUFAs (monounsaturated fatty acids) leads to increased high-density lipoprotein (HDL) cholesterol and decreased LDL cholesterol, triacylglycerols, lipid oxidation, and LDL susceptibility to oxidation (Kanu et al., 2007).

The Northeast of Portugal due to climatic conditions and flora diversity is one of the European regions with higher wild edible mushrooms diversity, some of them with great gastronomic relevance. In spite of the immense popularity of this food in the region and their increase exportation to foreign countries (particularly Spain, France and Italy), data regarding the nutritive value of the wild mushroom varieties available in the region are very meagre. The high nutritional quality and unique flavours of these mushrooms are likely to be lost if not documented. Therefore, it is now imperative that a nutritional database of these mushrooms is set up to retain the information on these unique species and for a better management and conservation of this natural resource and habitats related to them.

Herein, we intend to present a study of the chemical composition and biological properties of Portuguese wild mushrooms (*Cantharellus cibarius, Hypholoma fasciculare, Lepista nuda, Lycoperdon molle, Lycoperdon perlatum, Ramaria botrytis, Tricholoma acerbum*), in order to valorise these products as sources of nutrients and nutraceuticals. Chemical analyses included determination of proteins, fats, ash, and carbohydrates, and individual profiles in sugars, fatty acids and tocopherols by chromatographic techniques. Other compounds such as phenolics, flavonoids, carotenoids and ascorbic acid were also determined. Biological characterization was

focused in evaluation of mushrooms bioactive properties such as antioxidant and antimicrobial activities.

3.2.4.2. Materials and methods

Samples

Samples of *Cantharellus cibarius* L:Fries, *Lepista nuda* (Bull ex Fr) Cook, *Lycoperdon molle* (Pers.:Fr) Ricken, *Lycoperdon perlatum* Pers.:Pers., *Hypholoma fasciculare* (Huds) Quél, *Tricholoma acerbum* (Bull.:Fr) Quél, *Ramaria botrytis* (Pers.:Fr.) Ricken, were collected under live pine trees (*Pinus* sp.) for the first two species and under oak trees *(Quercus pyrenaica* Willd.) for the lasts species, in Bragança (Northeast of Portugal), in autumn 2006. Taxonomic identification was made according to several authors (Moser, 1983; Courtecuisse and Duhem, 1995) and representative voucher specimens were deposited at the herbarium of *Escola Superior Agrária* of *Instituto Politécnico de Bragança*. All the samples were lyophilised (Ly-8-FM-ULE, Snijders, Holland), reduced to a fine dried powder (20 mesh) and submitted to chemical composition and antioxidant activity analyses.

Standards and reagents

Acetonitrile 99.9%, n-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Lab-Scan (Lisbon, Portugal). All the other solvents were of analytical grade purity: methanol and diethyl ether were supplied by Lab-Scan, toluene was supplied by Riedel-de-Haën (Hanover, Germany), while sulphuric acid was supplied by Sigma Chemical Co. (St. Louis, MO, USA). The fatty acids methyl ester (FAME) reference standard mixture 37 (fatty acids C4 to C24; (standard 47885-U) was purchased from Sigma, as also other individual fatty acid isomers, tocopherol standards (α, β, γ and δ), and the standards used in the antioxidant activity assays: BHA (2-*tert*-butyl-4-methoxyphenol), TBHQ (tert-butylhydroquinone), L-ascorbic acid, α-tocopherol, gallic acid and (+)-catechin. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). The standards used in the antimicrobial activity assays, ampicillin and cycloheximide, as also butylated hydroxytoluene (BHT) and Folin and Ciocalteu's reagent were purchased from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

Chemical composition

Chemical parameters. Samples of mushrooms were analysed for chemical composition (moisture, protein, fat, carbohydrates and ash) as described in **3.1.1.2** (*Chemical composition*); reducing sugars were determined by dinitrosalicylic acid (DNS) method.

Fatty acids. Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GLC-FID)/capillary column based on the procedure described in **3.1.4.2** (*Fatty acid composition*).

Sugars. Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) based on the procedure described in **3.1.1.2** (*Sugar composition*).

Tocopherols. Quantification was based on the procedure described in **3.1.2.2** (*Extraction procedure* and *HPLC analysis*). Tocopherol contents in mushroom samples are expressed in ng per g of fresh mushroom.

Other bioactive compounds. Phenols, flavonoids, ascorbic acid and carotenoids were determined according to procedures described in chapter **3.1.4.2.** (*Total bioactive compounds*).

Biological properties

Samples preparation for antioxidant and antimicrobial activities assays. The samples (~3 g) were extracted by stirring with 100 mL of methanol at 25 ºC at 150 rpm for 24h and filtered through Whatman nº 4 paper. The residue was then extracted with two additional 100 mL portions of methanol, as described earlier. The combined methanolic extracts were evaporated at 40 ºC to dryness and redissolved in methanol for antioxidant activity assays or in DMSO for antimicrobial activity assays, at a concentration of 50 mg/mL, and stored at 4 ºC for further use.

Antioxidant activity

DPPH radical-scavenging activity, reducing power and inhibition of β-carotene bleaching were evaluated as described in **3.2.1.2**. Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS) was performed as described in **3.2.2.2**.

Antimicrobial activity

Microorganisms and culture conditions. Microorganisms labeled CECT were obtained from the Spanish type culture collection (CECT) of Valencia University, while microorganisms labeled ESA were clinically isolated strains from different biological fluids, identified by Microbiology Laboratory of *Escola Superior Agrária de Bragança*. Gram + (*Bacillus cereus* CECT 148, *B. subtilis* CECT 498, *Staphylococus aureus* ESA 7 isolated from pus) and Gram – (*Escherichia coli* CECT 101, *Pseudomonas aeruginosa* CECT 108, *Klebsiella pneumoniae* ESA 8 isolated from urine) bacteria, and fungi (*Candida albicans* CECT 1394, *Cryptococcus neoformans* ESA 3 isolated from vaginal fluid) were used to screen samples antimicrobial activity. Microorganisms were cultured aerobically at 37 ºC (Scientific 222 model) in nutrient agar medium for bacteria, and at 30 ºC in sabouraud dextrose agar medium for fungi.

Test assays for antimicrobial activity. A screening of antibacterial activities against the Gram - and Gram + bacteria and fungi was performed, according to the procedure described in **3.1.4.2** (*Test assays for antimicrobial activity*).

Statistical analysis

For each one of the mushroom species three samples were analysed and also all the assays were carried out in triplicate. The results were expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SAS v. 9.1.3 program.

3.2.4.3. Results and discussion

Chemical composition

The results of the chemical composition and estimated energetic value (expressed on fresh weight basis) obtained for the edible mushroom species are shown in **Table 3.2.8**. The moisture ranged from 88.65 g/100 g in *L. perlatum* and 93.77 g/100 g in *L. nuda*. Protein was found in high levels and varied between 1.83 g/100 g in *L. molle* and 4.09 g/100 g in *C. cibarius*. Fat ranged from 0.05 g/100g in *L. perlatum* and 0.22 g/100g in *C. cibarius*. The wild mushrooms were rich sources of protein and had low amounts of fat making them an ideal snack material, which is in agreement with other studies in different mushroom species (Longvah and Deosthale, 1998; Díez and Alvarez, 2001;

Agahar-Murugkar and Subbulakshmi, 2005). Carbohydrates, calculated by difference, were also an abundant macronutrient and ranged from 1.55 g/100 g in *L. nuda* and 6.80 g/100 g in *L. molle*. Reducing sugars are only a small part of carbohydrates content since polysaccharides such as chitin and starch, are the most abundant mushrooms carbohydrates (Manzi et al., 2001). Ash content varied between 0.88 g/100 g in *C. cibarius* and 3.62 g/100 g in *L. perlatum*. Being a good source of protein and carbohydrate, mushrooms prove to be excellent foods that can be used in low caloric diets for their low contents of fat and energy.

Among all the studied species, only *C. cibarius* chemical composition had already been described (Agahar-Murugkar and Subbulakshimi, 2005), but from a different country. Despite some similarities in the composition of Portuguese and Indian *C. cibarius* samples, it is known that the chemical composition of mushrooms is affected by a number of factors, namely mushroom strain/type, composition of growth media, time of harvest, management techniques, handling conditions, and preparation of the substrates (Manzi et al., 2001).

On the basis of the proximate analysis, it can be calculated that an edible portion of 100 g of these mushrooms assures, on average, 31 Kcal. The highest values are guaranteed by *R. botrytis*, while *L. nuda* give the lowest energy contribution (**Table 3.2.8**).

Table 3.2.8. Moisture, proximate chemical composition (g/100 g of fresh weight) and energetic value (Kcal/100 g of fresh weight) of the wild mushrooms (mean ± SD; n=3). In each column different letters mean significant differences ($p<0.05$).

Samples	Moisture	Total fat	Crude protein	Ash	Carbohydrates	Reducing sugars	Energy
Cantharellus cibarius	92.38 ± 0.31 ba	0.22 ± 0.04 a	4.09 ± 0.09 a	0.88 ± 0.05 c	2.44 ± 0.33 bc	0.26 ± 0.00 c	28.18 ± 1.39 ba
Lepista nuda	93.77 ± 0.98 a	0.11 ± 0.01 b	3.70 ± 0.61 b	1.15 ± 0.19 c	1.55 ± 0.98 c	0.10 ± 0.00 d	20.85 ± 4.68 b
Lycoperdon perlatum	88.65 ± 2.08 c	0.05 ± 0.01 d	1.94 ± 0.18 c	3.62 ± 0.37 a	5.74 ± 1.87 ba	0.44 ± 0.01 a	31.18 ± 6.90 ba
Lycoperdon molle	89.09 ± 1.27 bc	0.08 ± 0.02 cd	1.83 ± 0.04 c	2.20 ± 0.25 b	6.80 ± 1.57 a	0.42 ± 0.03 b	35.25 ± 6.01 a
Ramaria botrytis	89.77 ± 1.15 bc	0.14 ± 0.01 b	4.08 ± 0.18 a	0.90 ± 0.02 c	5.12 ± 1.02 ba	0.45 ± 0.00 a	38.03 ± 4.69 a

The results for fatty acid composition, total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) of the studied mushrooms are shown in **Table 3.2.9**.

Table 3.2.9. Fatty acid composition (percent) of the wild mushrooms (mean ± SD; n=3). Different letters mean significant differences ($p<0.05$).

	Cantharellus cibarius	*Lepista nuda*	*Lycoperdon molle*	*Lycoperdon perlatum*	*Ramaria botrytis*
C6:0	0.06 ± 0.01	0.09 ± 0.00	1.05 ± 0.06	0.56 ± 0.05	0.17 ± 0.05
C8:0	0.10 ± 0.00	0.02 ± 0.00	0.06 ± 0.00	0.05 ± 0.01	0.02 ± 0.00
C10:0	0.05 ± 0.00	0.10 ± 0.01	0.30 ± 0.02	0.15 ± 0.03	0.05 ± 0.00
C12:0	0.02 ± 0.00	0.11 ± 0.02	0.14 ± 0.01	0.15 ± 0.04	0.02 ± 0.00
C14:0	0.09 ± 0.00	0.33 ± 0.01	0.46 ± 0.01	0.40 ± 0.01	0.11 ± 0.01
C15:0	0.14 ± 0.00	0.79 ± 0.00	2.53 ± 0.06	1.86 ± 0.26	1.00 ± 0.01
C16:0	7.19 ± 0.14	11.77 ± 0.07	13.74 ± 0.17	12.90 ± 0.06	9.91 ± 0.03
C16:1	0.20 ± 0.00	0.51 ± 0.11	0.24 ± 0.00	0.32 ± 0.08	0.22 ± 0.03
C17:0	0.09 ± 0.00	*Nd*	0.90 ± 0.01	0.48 ± 0.08	0.70 ± 0.00
C18:0	3.34 ± 0.01	2.39 ± 0.00	2.35 ± 0.03	2.95 ± 0.01	2.36 ± 0.05
C18:1n9c	8.13 ± 0.01	29.53 ± 0.04	8.58 ± 0.07	4.59 ± 0.05	43.93 ± 0.14
C18:2n6c	50.01 ± 0.09	51.48 ± 0.11	64.15 ± 0.42	70.69 ± 0.87	38.32 ± 0.07
C18:3n3	0.10 ± 0.00	0.21 ± 0.00	0.06 ± 0.00	0.22 ± 0.05	0.02 ± 0.00
C20:0	0.18 ± 0.00	0.27 ± 0.01	0.36 ± 0.02	0.63 ± 0.02	0.13 ± 0.01
C20:1c	27.98 ± 0.20	0.05 ± 0.00	*nd*	*Nd*	0.44 ± 0.04
C20:2c	0.13 ± 0.01	0.09 ± 0.00	0.35 ± 0.01	*Nd*	0.28 ± 0.00
C20:3n6	*nd*	0.10 ± 0.01	*nd*	*Nd*	0.08 ± 0.00
C20:3n3+C21:0	0.12 ± 0.01	0.07 ± 0.00	0.53 ± 0.09	0.27 ± 0.06	0.04 ± 0.00
C20:5n3	0.09 ± 0.00	0.15 ± 0.00	0.82 ± 0.02	0.33 ± 0.02	0.16 ± 0.01
C22:0	0.23 ± 0.01	0.55 ± 0.02	1.22 ± 0.02	1.32 ± 0.27	0.86 ± 0.04
C23:0	0.06 ± 0.00	0.29 ± 0.01	0.50 ± 0.03	0.48 ± 0.03	0.17 ± 0.01
C24:0	0.51 ± 0.02	0.78 ± 0.08	1.43 ± 0.01	1.64 ± 0.06	0.88 ± 0.06
C24:1	1.23 ± 0.04	0.21 ± 0.02	0.23 ± 0.05	*Nd*	0.10 ± 0.01
total SFA	12.04 ± 0.52 e	17.58 ± 0.08 c	25.04 ± 0.27 a	23.57 ± 0.52 b	16.38 ± 0.06 d
total MUFA	37.54 ± 0.14 b	30.32 ± 0.02 c	9.04 ±0.01 d	4.91 ± 0.14 e	44.69 ± 0.07 a
total PUFA	50.42 ± 0.65 d	52.10 ± 0.10 c	65.92 ± 0.25 b	71.52 ± 0.65 a	38.91 ± 0.02 e

nd- not detected.

In general, the major fatty acid found in the studied samples was linoleic acid (C18:2), followed by oleic acid (C18:1) and palmitic acid (C16:0). In fact, it is known that linoleic acid is the precursor of 1-octen-3-ol, known as the alcohol of fungi, which is the principal aromatic compound in most fungi and might contribute to mushrooms flavour (Maga, 1981). Besides the three main fatty acids already described, twenty more were identified and quantified. PUFA were the main group of fatty acids in all species with exception for *R. botrytis*, where MUFA were the main group. In general, UFA predominated over SFA for all the studied mushroom species, ranging from 75 to 88%. This is consistent with previous observations (Longvah and Deosthale, 1998; Díez and

Alvarez, 2001). Considering total MUFA content, *L. perlatum* had the lowest value but contained the highest PUFA content, due to the higher contribution of linoleic acid. *L. molle* had the highest SFA value, due to the presence of higher amounts of palmitic acid. *Trans* isomers of unsaturated fatty acids were not detected in the studied mushrooms; some of these compounds have been related to increased risk of cardiovascular disease being negatively correlated with plasma HDL-cholesterol concentration and positively correlated with plasma LDL-cholesterol level (Kanu et al., 2007).

In what concerns sugar composition (**Table 3.2.10**) the edible mushrooms presented mannitol and trehalose as main sugars. For *C. cibarius* (1.06 g/100 g) and *R. botrytis* (1.20 g/100 g) mannitol was the most abundant sugar, while trehalose predominated in *L. nuda*, *L. perlatum* and *L. molle*, ranging from 0.17 g/100 g to 0.75 g/100g. The accumulation of these sugars in the fruit-bodies of other species was already reported (Decker, 1997; Harada et al., 2004). Nevertheless, the present study describes for the first time the presence of maltose (disaccharide) and melezitose (non-reducing trisaccharide).

Table 3.2.10. Sugar composition (g/100 g of fresh weight) of the wild mushrooms (mean ± SD; n=3). In each column different letters mean significant differences ($p<0.05$).

	Mannitol	Trehalose	Maltose	Melezitose	Total sugars
Cantharellus cibarius	1.06 ± 0.02 b	0.85 ± 0.01 a	*nd*	*Nd*	1.91 ± 0.03 a
Lepista nuda	0.05 ± 0.00 c	0.75 ± 0.03 b	0.10 ± 0.00 a	*Nd*	0.85± 0.03 c
Lycoperdon perlatum	0.02 ± 0.00 d	0.29 ± 0.01 c	*nd*	*Nd*	0.31 ± 0.01 d
Lycoperdon molle	*Nd*	0.17 ± 0.05 d	*nd*	*Nd*	0.17 ± 0.05 e
Ramaria botrytis	1.20 ± 0.01 a	0.20 ± 0.01 d	*nd*	0.02 ± 0.00 a	1.42 ± 0.00 b

nd- not detected.

Overall, *R. botrytis* revealed the highest energetic value, but with the highest protein content. It also presented the highest MUFA and the lowest SFA levels, which may be relevant since the substitution of SFA with MUFA leads to an increase in HDL cholesterol and decrease in LDL cholesterol, triacylglycerol, lipid oxidation, and LDL susceptibility to oxidation (Kanu et al., 2007). Concerning sugar composition it presented the highest percentage of mannitol, which functions to provide support and expansion of the fruit body as other sugar alcohols. *Ramaria botrytis* is one of the largest of the coral fungi and is considered an excellent edible by some mycophagists.

The tocopherols content was determined in seven Portuguese mushroom species (**Table 3.2.11**), including five edible samples (nutritional composition above described) but also two non-edible species (*Hypholoma fasciculare* and *Tricholoma acerbum*).

Table 3.2.11. Tocopherol composition (ng/ g of fresh weight) of the wild mushrooms (mean ± SD; n=3). In each column different letters mean significant differences ($p<0.05$).

	α-tocopherol	β-tocopherol	γ-tocopherol	Total
Cantharellus cibarius	13.40 ± 0.76 dc	2.87 ± 0.06 e	*nd*	16.27 ± 0.76 e
Hypholoma fasciculare	16.06 ± 0.08 dc	40.46 ± 0.40 b	25.69 ± 0.45 a	82.20 ± 0.63 b
Lepista nuda	7.95 ± 0.49 d	12.13 ± 0.37 c	14.64 ± 0.18 b	34.72 ± 0.43 c
Lycoperdon molle	27.13 ± 0.42 a	*nd*	*nd*	27.13 ± 0.42 dc
Lycoperdon perlatum	25.23 ± 0.76 ba	*nd*	*nd*	25.23 ± 0.76 de
Ramaria botrytis	20.54 ± 0.88 bc	229.81 ± 0.99 a	*nd*	250.35 ± 1.35 a
Tricholoma acerbum	13.73 ± 0.29 dc	6.53 ± 0.49 d	2.79 ± 0.17 c	23.05 ± 0.92 de

nd.- not detected

The results obtained in the analysis of mushroom samples point to the existence of differences in what concerns tocopherols composition among different species. α-Tocopherol was found in all the species being the major compound for *C. cibarius*, *L. molle*, *L. perlatum* and *T. acerbum*; β-tocopherol was the major compound for *R. botrytis* and *H. fasciculare*, while γ-tocopherol was the major compound in *L. nuda*. *R. botrytis* presented the higher content of tocopherols (250.35 ng/g of fresh weight) while *C. cibarius* revealed the lowest content (16.27 ng/g). Some authors published tocopherols determination in other mushrooms, but using a different methodology (Elmastas et al., 2007; Tsai et al., 2007). In the present work we did not performed a saponification step in the samples preparation, since saponification is more time-consuming and laborious, eventually conducting to tocopherol degradation due to their high sensivity to light, heat and oxygen. We also introduced an antioxidant protector to minimize tocopherols loss. In previous study, these modifications were shown to provide a precise and accurate determination of tocopherols (see chapter 3.1.2). α-Tocopherol was already determined by Elmastas *et al*. (2007) in *L. nuda* from Turkey, but the results were expressed per mg of extracts.

Table 3.2.12 presents phenolics, flavonoid, ascorbic acid and carotenoids concentrations obtained in the mushroom extracts. Phenolic compounds were the major antioxidant components found in the extracts (1.75-20.32 mg/g), followed by flavonoids

(0.47-16.56 mg/g). Ascorbic acid was found in small amounts (0.09–0.40 mg/g), and β-carotene and lycopene were only found in vestigial amounts (<0.08 mg/g). *R. botrytis* revealed a higher content in phenol and flavonoid compounds, which significantly (p<0.05) decreased in the other species.

Table 3.2.12. Total bioactive compounds of the wild mushrooms (mean ± SD; n=3). In each column different letters mean significant differences (p<0.05).

	Phenolics (mg/g)	Flavonoids (mg/g)	Ascorbic acid (mg/g)	β-carotene (μg/g)	Lycopene (μg/g)
Cantharellus cibarius	1.75 ± 0.50 e	0.47 ± 0.05 f	0.40 ± 0.02 a	5.77 ± 0.41 e	1.95 ± 0.28 d
Hypholoma fasciculare	17.67 ± 0.27 b	5.09 ± 0.48 b	0.09 ± 0.00 e	24.62 ± 0.76 b	11.90 ± 0.51 b
Lepista nuda	6.31 ± 0.13 d	3.36 ± 0.50 c	0.23 ± 0.03dc	2. 52 ± 0.25 g	0.98 ± 0.13 e
Lycoperdon molle	11.48 ± 0.52 c	2.45 ± 0.08 d	0.34 ± 0.08 b	4.48 ± 0.23 f	2.19 ± 0.15 d
Lycoperdon perlatum	10.57 ± 0.17 c	2.10 ± 0.14 ed	0.21 ± 0.02 d	12.50 ± 0.53 c	6.39 ± 0.34 c
Ramaria botrytis	20. 32 ± 1.87 a	16.56 ± 0.30 a	0.27 ± 0.04 c	10.41 ± 0.48 d	1.51 ± 0.22 ed
Tricholoma acerbum	5.53 ± 0.63 d	1.87 ± 0.71 e	0.22 ± 0.04 dc	75.48 ± 2.01 a	39.65± 1.33 a

Biological properties

The bioactive properties (antioxidant and antimicrobial) were evaluated using the whole extract, which is a complex mixture of phytochemicals with possible additive and synergistic effects. To screen the antioxidant properties, several chemical and biochemical assays using animal cells were performed: reducing power, scavenging activity on DPPH radicals, inhibition of β-carotene bleaching, and inhibition of lipid peroxidation in brain tissue. All the species proved to have antioxidant activity (**Table 3.2.13**) being more significant for *R. botrytis* (lower EC_{50} values). *C. cibarius* presented the lowest antioxidant properties (higher EC_{50} values) which are compatible to its lower phenolics and tocopherols content. These properties seem to be related to total phenolic and tocopherols contents. The bioactivity of phenolics may be related to their ability to chelate metals, inhibit lipoxygenase and scavenge free radicals (Decker, 1997). Tocopherol functions as a chain-breaking antioxidant for lipid peroxidation in cell membranes and also as a scavenger of ROS such as singlet oxygen (Kanu et al., 2007).

Table 3.2.13. EC_{50} values (mg/mL) obtained for the antioxidant activity of the wild mushrooms (mean ± SD; n=3). In each column different letters mean significant differences ($p<0.05$).

	DPPH scavenging activity	Reducing power	β-carotene bleaching inhibition	Lipid peroxidation inhibition
Cantharellus cibarius	19.65 ± 0.28 a	8.72 ± 0.03 a	8.40 ± 0.87 a	8.59 ± 0.73 a
Hypholoma fasciculare	1.13 ± 0.03 f	0.95 ± 0.01 f	0.86 ± 0.02 f	1.55 ± 0.53 e
Lepista nuda	4.41 ± 0.01 b	3.53 ± 0.09 b	4.21 ± 0.09 c	5.80 ± 0.07 b
Lycoperdon molle	3.23 ± 0.09 e	2.27 ± 0.00 e	1.92 ± 0.05 e	3.31 ± 0.23 d
Lycoperdon perlatum	3.95 ± 0.04 c	2.96 ± 0.01 d	2.49 ± 0.06 d	4.64 ± 1.40 c
Ramaria botrytis	0.66 ± 0.00 g	0.68 ± 0.00 g	0.67 ± 0.01 f	1.01 ± 0.02 e
Tricholoma acerbum	3.60 ± 0.08 d	3.27 ± 0.02 c	5.89 ± 0.28 b	6.20 ± 0.67 b

Table 3.2.14 shows the antimicrobial screening of mushroom extracts against *B. cereus*, *B. subtilis*, *S. aureus* (Gram +), *E. coli*, *P. aeruginosa*, *K. peumoniae* (Gram -) bacteria, and *C. albicans* and *C. neoformans* (fungi). *Lycoperdon* spp extracts showed no effect agains any of the tested microorganisms, whilst the other samples revealed antimicrobial activity selectively against Gram + bacteria, with very low MICs. Particularly, *C. cibarius* and *L. nuda* presented MIC values against *B. subtilis* and *S. aureus* even lower than the standard ampicillin. Despite the existing of some studies demonstrating that phenolic compounds possess antimicrobial activity (Puupponen-Pimiä et al., 2001), herein a relation between phenolic content and antimicrobial properties seemed not to exist. In fact, there are other compounds, such steroids, oxalic acid, sesquiterpenoids, and epipolythiopiperazine-2,5-diones isolated from mushrooms that proved to have antimicrobial activity (Lindequist et al., 2005).

Table 3.2.14. Antimicrobial activity of the wild mushrooms (mean ± SD; n=3).

	MIC (μg/mL)							
Samples	*B. cereus*	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*E. coli*	*K. peumoniae*	*C. albicans*	*C. neoformans*
Cantharellus cibarius	5 (++++)	5 (++++)	5 (++++)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)
Hypholoma fasciculare	500 (+++)	5 (++++)	500 (++++)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)
Lepista nuda	5 (++++)	5 (++++)	5 (++++)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)
Lycoperdon molle	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)
Lycoperdon perlatum	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)
Ramaria botrytis	50 (++++)	50 (++++)	5 (++++)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)
Tricholoma acerbum	500 (-)	500 (++++)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)	500 (-)
Ampicillin	3.13 (++++)	12.5 (++++)	6.25 (++++)	6.25 (++++)	6.25 (++++)	6.25 (++++)	NT	NT
Cycloheximide	NT	NT	NT	NT	NT	NT	12.5 (++)	6.25 (++++)

No antimicrobial activity at the higher tested concentration (-), inhibition zone < 1 mm. Slight antimicrobial activity (+), inhibition zone 2-3 mm. Moderate antimicrobial activity (++), inhibition zone 4-5 mm. High antimicrobial activity (+++), inhibition zone 6-9 mm. Strong antimicrobial activity (++++), inhibition zone > 9 mm. Standard deviation ± 0.5 mm. NT. not tested.

The edibility seems not to be related to bioactive properties, since one of the non-edible mushrooms (*H. fasciculare*) revealed high antioxidant and antimicrobial activities and high phenols and tocopherols contents, while the other one (*T. acerbum*) presented lower bioactive properties.

In conclusion, the analysed mushrooms contain very useful phytochemicals such as phenolics, tocopherols, ascorbic acid and carotenoids, and revealed interesting antioxidant and antimicrobial properties. The combination of their bioactive compounds and rich nutritional composition (high contents in protein and carbohydrates, low contents in fat with the precious contribution of unsaturated fatty acids, and absence of *trans* fatty acids) makes them very special. This study aims to contribute not only to a better knowledge of the product but also to its valorisation.

3.3. Antioxidant and antimicrobial properties of mushrooms mycelium

3.3.1. Influence of the culture medium and pH on the growth of saprobic and ectomycorrhizal mushroom mycelia

3.3.1.1. Introduction

Mushrooms have become attractive as a functional food and as sources for the development of drugs and nutraceuticals (Chang, 1996; Chang 1999), namely for their antioxidant compounds (Yen and Hung, 2000; Mau et al., 2002a; Yang et al., 2002; Cheung et al., 2003; Cheung and Cheung, 2005; Lo and Cheung, 2005). In addition to dried mushrooms, alternative or substitute mushroom products are mycelia that could also be used as food and food-flavouring material, or in the formulation of nutraceuticals and functional foods. The nutritional value and taste components of some mushroom mycelia have been studied (Weng, 2003).

The Northeast of Portugal, due to their climatic conditions and flora diversity, is one of the European regions with higher wild edible mushrooms diversity, some of them with great gastronomic relevance. *Lactarius deliciosus* (L.) Gray, *Suillus luteus* (L.) Gray and *Leucopaxillus giganteus* (Sowerby) Singer are important local edible species for their high harvesting and consumption in the rural population, and also due to their commercialization and economic value in the international markets, as Spain, France and Italy (Martins et al., 2002; Baptista et al., 2005). These *Basidiomycete* fungi belong to the order *Russulales*, *Boletales* and *Agaricales*, and to the families *Russulaceae*, *Boletaceae* and *Tricholomataceae*, respectively. Saprobic *Leucopaxillus* species have been reported for their medicinal activity, being used in chemical industry for extraction of clitocybin antibiotic. The other mushrooms are ectomycorrhizal (associations between fungi and some plant roots).

The growth of saprobic edible mushrooms with their fruiting body as the most common edible form is a lengthy and complex process involving the use of solid compost or lignocellulosic waste, such as straw or cotton, followed by a long cultivation period (Vedder 1978). Ectomycorrhizal mushroom fruiting bodies could not be grown at all to data. Therefore, growing mushroom mycelium on a defined nutrient medium could be an alternative method to produce fungal biomass (Cirrillo et al., 1960; Litchfield, 1967;

Cheung, 1995), being the culture conditions vital for enhancing the efficiency of mycelia growth.

In this study, the influence of different culture media and pH values on the radial growth and growth rate of *Leucopaxillus giganteus*, *Lactarius deliciosus* and *S. luteus* mycelium has been evaluated. The mycelium growth was weekly followed during 80 days for ectomycorrhizal mushrooms and 24 days for the saprobic mushroom, and morphological characteristics were also evaluated for each tested conditions.

3.3.1.2 Materials and methods

Samples

Leucopaxillus giganteus (Sowerby) Singer was collected in grassland whereas *Lactarius deliciosus* (L.) Gray and *Suillus luteus* (L.) Gray were collected under living pine trees (*Pinus* sp.), in Bragança (Northeast of Portugal), in autumn 2005. Taxonomic identification was made according to several authors (Marchand, 1971-1986; Moser, 1983; Bon, 1988; Courtecuisse and Duhem, 1995; Courtecuisse, 1999) and liophylized (Ly-8-FM-ULE, Snijders, Holland). Representative voucher specimens were deposited at the herbarium of *Escola Superior Agrária* of *Instituto Politécnico de Bragança*.

Isolation of the biological material

Mycelia of *Leucopaxillus giganteus*, *Lactarius deliciosus* and *S. luteus* were isolated from sporocarps, on agar Melin-Norkans (MMN) medium (NaCl 0.025 g/L; $(NH_4)_2HPO_4$ 0.25 g/L; KH_2PO_4 0.50 g/L; $FeCl_3$ 0.050 g/L; $CaCl_2$ 0.50 g/L; $MgSO_4.7H_2O$ 0.15 g/L; thiamine 0.10 g/L; casaminoacid 1.0 g/L; malt extract 10 g/L; glucose 10 g/L; agar 20 g/L), following Brundrett et al. (1996). The strains were maintained in Petri dishes (9 cm diameter) containing the same medium at 25ºC in the dark and sub-cultured every two weeks.

Effect of culture medium and pH on mycelia growth

The effect of culture medium and pH on mycelia growth of the studied mushrooms was performed in Petri dishes (9 cm diameter) containing 10 mL of solid medium. The following different nutritive solid medium were tested: Melin-Norkans (MMN) medium pH 5 or 6, and Potato Dextrose Agar (PDA: potato 4 g/L; dextrose 20g/L; agar 15 g/L) pH 5 or 6. Inoculation of Petri dishes was performed with hyphal plugs (created with a

pipette tip, 5 mm diameter) of 2-week-old mycelia (one plug per plate). Five replicates for each medium were performed. The cultures were incubated at 25ºC in the dark. Fungal growth (colony radius) was measured weekly at four right angles after 80 days of inoculation. In the case of *Leucopaxillus giganteus* the growth was followed in smaller periods (3 days) until 24 days of inoculation.

Mycelia morphological description

The morphological description of mycelia growth in the different nutritive solid media was weekly performed. The parameters recorded were: colony texture and colour, border appearance and colour, reverse colour, medium coloration, aerial growth, exudates and rifts.

Statistical analysis

Each experiment was carried out using five Petri dishes (four colony radius values in each one) and the results are expressed as mean values and standard error. Differences among means were done by analysis of variance (ANOVA), using SAS v. 9.1.3, and averages were compared using Tukey test ($p < 0.05$).

3.3.1.3. Results and discussion

Effect of culture medium and pH on mycelia growth

The mycelium growth in different solid nutritive culture media (MMN or PDA) and at different pH values (5 or 6) is shown in **Table 3.3.1.**

In general, the mycelium growth varied either with the culture medium or pH medium. For *Lactarius deliciosus*, MMN proved to be a better culture medium than PDA. Although the pH had no influence on the mycelium growth using MMN medium, for PDA culture medium pH 5 was significantly ($p<0.05$) better than pH 6. The growth of *L. deliciosus* mycelium in all the tested culture conditions significantly ($p<0.05$) increased till the end of the experiment.

For *S. luteus*, the use of MMN culture medium instead of PDA significantly ($p<0.05$) increased the mycelium growth and once more, pH had no influence on the mycelium growth using this medium. In the case of PDA culture medium, pH 5 was only significantly ($p<0.05$) better than pH 6 between 58 and 80 days of growth. The growth

of *S. luteus* mycelium in MMN medium significantly (p<0.05) increased during the whole experiment, while culture radius in PDA only increased after 58 days.

Table 3.3.1. Mean radial growth (cm) of *Lactarius deliciosus*, *Suillus luteus* and *Leucopaxillus giganteus* cultures, in the presence of different nutritive culture medium (MMN or PDA) and pH values (5 or 6), along time of inoculation.

	Radial growth[a] (cm)			
Days after inoculation	MMN pH 5	MMN pH 6	PDA pH 5	PDA pH 6
	Lactarius deliciosus			
7	0	0	0	0
14	0.28±0.07 a	0.25±0.08 a	0.16±0.09 b	0 c
21	0.51±0.07 a	0.47±0.07 a	0.36±0.11 b	0.18±0.07 c
28	0.80±0.09 a	0.75±0.11 a	0.55±0.16 b	0.18±0.07 c
36	1.13±0.14 a	1.29±0.15 a	0.74±0.17 b	0.23±0.12 c
43	1.59±0.22 a	1.45±0.24 a	0.94±0.17 b	0.38±0.09c
51	2.03±0.34 a	2.05±0.42 a	1.11±0.24 b	0.48±0.10 c
58	2.55±0.42 a	2.61±0.52 a	1.13±0.18 b	0.55±0.13 c
64	2.92±0.44 a	2.94±0.52 a	1.16±0.22 b	0.62±0.12 c
72	3.36±0.41 a	3.40±0.49 a	1.23±0.22 b	0.80±0.16 c
80	3.67±0.44 a	3.70±0.37 a	1.31±0.10 b	1.08±0.33 b
	Suillus luteus			
7	0.06±0.12 ba	0.07±0.09 a	0 b	0 b
14	0.31±0.10 a	0.28±0.17 a	0.13±0.11 b	0.10±0.08 b
21	0.31±0.10 a	0.33±0.20 a	0.13±0.11 b	0.10±0.08 b
28	0.61±0.10 a	0.63±0.37 a	0.25±0.16 b	0.15±0.14 b
36	0.88±0.20 a	0.93±0.60 a	0.26±0.16 b	0.18±0.20 b
43	1.73±0.47 a	1.51±0.88 a	0.54±0.27 b	0.22±0.19 b
51	2.06±0.63 a	1.81±1.06 a	0.70±0.35 b	0.22±0.19 b
58	2.37±0.83 a	2.37±0.83 a	0.87±0.44 b	0.25±0.19 c
64	2.73±0.93 a	2.49±1.12 a	1.21±0.53 b	0.41±0.19 c
72	3.07±1.03 a	2.65±1.13 a	1.68±0.63 b	0.55±0.24 c
80	3.29±1.09 a	2.93±1.13 a	2.01±0.70 b	0.84±0.42 c
	Leucopaxillus giganteus			
7	0.56±0.15 b	1.67±0.37 a	1.66±0.09 a	1.42±0.43 a
10	0.78±0.22 b	2.37±0.35 a	2.32±0.59 a	2.13±0.59 a
14	1.44±0.51 b	3.40±0.24 a	3.06±0.19 a	3.19±0.59 a
17	2.08±0.77 c	3.91±0.23 a	3.38±0.22 b	3.67±0.41 ba
21	2.95±0.93 b	3.97±0.23 a	3.71±0.26 a	3.92±0.29 a
24	3.46±0.85 b	4.01±0.19 a	3.81±0.21 ba	3.97±0.24 a

[a]Each value is expressed as mean ± standard deviation (n=5). In each row different letters mean significant differences (p <0.05).

The growth of *L. giganteus* in PDA culture medium at both pH and in MMN at pH 6 was very similar (p>0.05) until 14 days. After 17 days of growth differences were significant (p<0.05) and in the order MMN, pH 6 > PDA, pH 6 > PDA, pH 5. When MMN at pH 5 was used the mycelium growth was significantly (p<0.05) lower in comparison to pH 6. The growth of this mushroom mycelium only significantly

increased ($p<0.05$) until 21 and 17 days of growth for pH 5 and 6, respectively. After these points and till the end of the experiments the radial growth did not increase.

After 24 days, *L. giganteus* (saprobic) mycelium had grown for all the Petri dish, while the growth of *L. deliciosus* and *S. luteus* (ectomycorrhizal) had to be followed until 80 days.

In **Figure 3.3.1** we present the mycelia growth rate in different culture media and at different pH values. For all the tested conditions, the growth rate of *Leucopaxillus giganteus* mycelium (eg. 166.90 ± 0.19 mm/day in MMN6) was significantly higher ($p<0.05$) than the growth rate of ectomycorrhizal mushroom mycelium (46.22 ± 0.37 mm/day for *Lactarius deliciosus* and 36.65 ± 1.13 mm/day for *S. luteus*). The growth rate was very similar for *Lactarius deliciosus* and *S. luteus* being MMN the best culture medium.

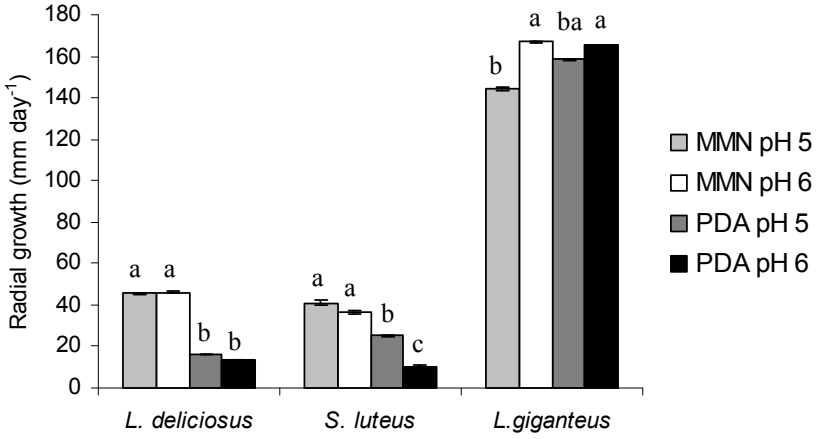


Figure 3.3.1. Mean radial growth rate (mm day^{-1}) of *Lactarius deliciosus*, *Suillus luteus* and *Leucopaxillus giganteus* cultures, in the presence of different nutritive culture medium (MMN or PDA) and pH values (5 or 6). Each value is expressed as mean ± standard error (n=5). Different letters above column groups indicate significantly different means at $p<0.05$.

Mycelia morphological description

Morphological description of the mycelia grown in different culture media and at different pH values is presented in **Table 3.3.2**. The pH had no influence on the mycelial morphological characteristics, while the culture medium only affected *S. luteus*.

L. deliciosus mycelium grown under the different conditions was beige and woolly, unless for PDA medium at pH 6 in which it became cottony. The border was white and diffuse changing to clear only in PDA 6 (**Figure 3.3.2**). The reverse in MMN and PDA

culture media was yellowish and yellow/green, respectively. It was not observed any aerial growth, medium coloration, exudates or rifts.

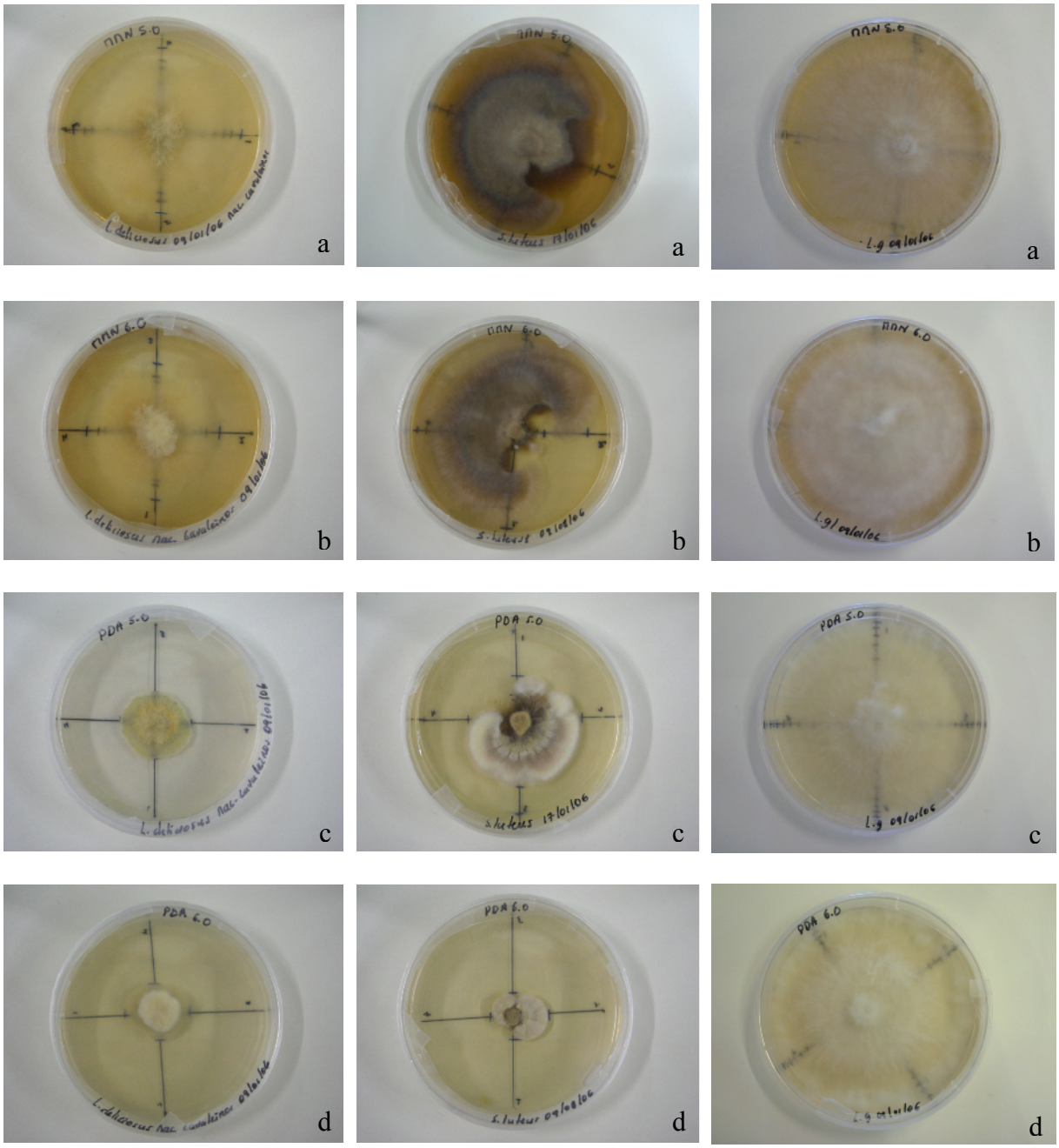
S. luteus mycelium grown in MMN culture medium was whitish brown and furry, changing to brownish white and cottony in PDA culture medium. In both cases, the border was white and clear (**Figure 3.3.2**). The reverse was brown and brownish orange for MMN and PDA culture medium, respectively. It was observed aerial growth, and in the case of PDA culture the medium was also coloured.

Table 3.3.2- Morphological description of *L. deliciosus*, *S. luteus* and *L. giganteus* grown in different nutritive culture media and at different pH values.

Species	Culture medium	pH	Mycelium texture	Mycelium colour	Border	Border colour	Reverse colour	Aerial growth	Medium coloration	Exudates	Rifts
L. deliciosus	MMN	5.0	Wooly	Beige	Diffuse	white	yellowish	-	-	-	-
		6.0	Wooly	Beige	Diffuse	white	yellowish	-	-	-	-
	PDA	5.0	Wooly	Beige	Diffuse	white	yellow/green	-	-	-	-
		6.0	Cottony	Beige	Clear	white	yellow/green	-	-	-	-
S.luteus	MMN	5.0	furry[a]	whitish brown[b]	Clear	white	brown	+	-	-	-
		6.0	furry[a]	whitish brown[b]	Clear	white	brown	+	-	-	-
	PDA	5.0	Cottony	brownish white[b]	Clear	white	brownish orange	+	+	-	-
		6.0	Cottony	brownish white[b]	Clear	white	brownish orange	+	+	-	-
L. giganteus	MMN	5.0	Cottony	White	Diffuse	white	white	+	-	-	-
		6.0	Cottony	White	Diffuse	white	white	+	-	-	-
	PDA	5.0	Cottony	White	Diffuse	white	white	+	-	-	-
		6.0	Cottony	White	Diffuse	white	white	+	-	-	-

[a] cottony until 43 days of growth; [b] white until 21 days of growth

L. giganteus presented the same characteristics in both culture media and pH values (**Figure 3.3.2**). The mycelium was white and cottony, the border was white and diffuse, and the reverse was also white. In all the cases, it was possible to observe aerial growth.



Lactarius deliciosus *Suillus luteus* *Leucopaxillus giganteus*

Figure 3.3.2. Appearance of *Lactarius deliciosus, Suillus luteus* (after 80 days of growth) and *Leucopaxillus giganteus* (after 24 days of growth) colonies, in the presence of different nutritive culture medium and pH values: (a) MMN pH 5, (b) MMN pH 6, (c) PDA pH 5, (d) PDA pH 6.

Overall, the pH had no influence in mycelia morphological characteristics, and the culture medium only affected *S. luteus* mycelium characteristics. For all the tested conditions, the growth rate of *Leucopaxillus giganteus* mycelium was significantly higher than the growth rate of ectomycorrhizal mushrooms mycelium. For *Lactarius deliciosus* and *S. luteus* MMN proved to be the better nutritive medium; it has already been referred to as the medium that usually offers the best results for ectomycorrhizal fungi (Marx and Kenney, 1982; Molina and Palmer, 1982). It is also known that pH culture medium influence the fungi growth. Although some studies reported considerable growth values between pH 3.2 and 6.5, the optimal pH ranges between 4.5 and 5.5 as optimum pH ranges, which are related to enzymatic systems, essential vitamin entry in the cell, surface metabolic reactions and mineral capture. Some fungi, however, are able to adjust to the pH of the medium, optimising it for their better development (Hung and Trappe, 1983; Torres and Honrubia, 1991). In our study the pH had a significantly influence in the growth of *Leucopaxillus giganteus* and *S. luteus* when PDA medium was used. Nevertheless, the results of the in vitro assays testing the pH effect on fungi growth should be carefully analysed since they may be influenced by the incubation time, nitrogen source and iron salts addition before or after the medium sterilization (Hung and Trappe, 1983).

The results obtained imply that the mycelium growth and appearance not only varies with the culture medium and/or pH value, but also changes with the mushroom species, being the saprobic mycelium growth rate significantly higher than the growth rate of ectomycorrhizal mushrooms mycelium.

3.3.2. Bioactive properties of the medicinal mushroom *Leucopaxillus giganteus* mycelium produced in the presence of different nitrogen sources

3.3.2.1. Introduction

Mushrooms have become attractive as functional foods and as sources for the development of drugs and nutraceuticals (Yang et al., 2002; Mau et al., 2004; Cheung and Cheung, 2005). In addition to dried mushrooms, alternative or substitute mushroom products are mycelia that could also be used as food and food-flavouring material, or in the formulation of nutraceuticals and functional foods. The add-value arising from mushrooms/mycelia bioactive properties can leave to an increase in its consumption and therefore stimulating the commercialisation of local edible species and the *in vitro* production of mycelium mushroom (e.g., for pharmaceutical industry). Over two-thirds of cancer-related death could be prevented through lifestyle modification, particularly through dietary means, and mushrooms consumption could contribute to minimize cancer risks through antioxidants input (Borchers et al., 2004).

Leucopaxillus species have received extensive attention for medical application, being used in chemical industry for extraction of clitocybin antibiotic (Breitenbach and Kränzlin, 1991). *Leucopaxillus giganteus* (Sowerby) Singer is a common Portuguese edible mushroom and belongs to the phyla *Basidiomycete*, order *Agaricales*, and family *Tricholomataceae*. However, it is difficult and time-consuming to cultivate the fruiting bodies of *L. giganteus* for obtaining bioactive compounds. At present, between 80 and 85% of all medicinal mushroom products are derived from the fruiting bodies, which have been either commercially farmed or collected from the wild. Only 15% of all products are based on extracts from mycelia (Lindequist et al., 2005). In spite of a great need for useful bioactive metabolites production by submerged cultivation of mushrooms, the bioprocess development is still far from being thoroughly studied (Cho et al., 2002; Wasser et al., 2003; Zhong and Tang, 2004). Usually, culture medium is important to the yield of any cultivation products and nitrogen source generally plays a significant role, as it is essential for cell proliferation and metabolite biosynthesis. In submerged cultivation of mushroom *Tremella mesenterica*, Wasser et al. (2003) demonstrated that type and concentration of nitrogen sources strongly influenced cell growth and polysaccharide production. Studies have been carried out on the effect of nitrogen source on growth of edible mushrooms, particularly on Nigerian species

Vovariella speciosa (Fr. Ex. Fr.) Sing. (Fasidi and Akwakwa, 1996), *Psathyerella atroumbonata* (Pegler) (Jonathan and Fasidi, 2001), *Lentinus subnudus* (Berk.) (Gbolagade et al., 2006), Chinese species *Pleurotus tuber-regium* (Fr.) (Wu et al., 2003; Wu et al., 2004), *Cordyceps militaris* (Mao and Zhong, 2006) and Brazilian species *Agaricus brasiliensis* (*A. blazei*) (Fan et al., 2007), however, no studies are known in Portuguese species and no reports are known about the influence of the nitrogen source in the bioactive properties of wild edible mushrooms. In this work, the effects of various nitrogen sources on the production and functional properties of *Leucopaxillus giganteus* mycelium are evaluated. The mycelium growth was followed along the time during 60 days and the antimicrobial activity was screened using different microorganisms, namely Gram positive (*Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*) and Gram negative (*Pseudomonas aeruginosa*, *Escherichia coli, Klebsiella penumoniae*) bacteria, and fungi (*Candida albicans*, *Cryptococcus neoformans*) provided by collection strains or clinical isolates. The antioxidant activity was evaluated for each nitrogen source and for each growth day, using several assays: free radical scavenging capacity, reducing power, oxidative erythrocytes hemolysis inhibition and lipid peroxidation inhibition. All these antioxidant activity parameters were correlated to the phenolic and flavonoidic contents in the samples.

3.3.2.2.Materials and methods

Samples

Mycelia of *L. giganteus* (Sowerby) Singer was isolated from sporocarps collected under grassland in Bragança (Northeast of Portugal) (Herbarium of Agrarian School - *Instituto Politécnico Bragança*) on solid Melin-Norkans (MMN) medium pH 6.6 (NaCl 0.025 g/L; $(NH_4)_2HPO_4$ 0.25 g/L; KH_2PO_4 0.50 g/L; $FeCl_3$ 0.050 g/L; $CaCl_2$ 0.50 g/L; $MgSO_4.7H_2O$ 0.15 g/L; thiamine 0.10 g/L; casaminoacid 1.0 g/L; malt extract 10 g/L; glucose 10 g/L; agar 20 g/L), following Brundrett et al. (1996). The strain was maintained in the same medium at 25ºC in the dark and sub-cultured every month.

Effect of nitrogen source on mycelia growth rate

For aseptic establishment of assay, 10 hyphal plugs (5 mm diameter) of 1-week-old *L. giganteus* mycelia were transferred into flasks (700 mL) containing 250 mL of MMN liquid medium prepared with four different nitrogen sources: potassium nitrate,

ammonium nitrate, biammonium phosphate and sodium nitrite. Inoculated flasks were shaked and maintained in the dark at 25ºC. After 15, 30, 45 and 60 days of growth the mycelium was recovered from the liquid medium by filtration, washed with distilled water, weighted (fw) and dried at 50ºC, during 24h, to obtain the yield of biomass (dw). Three replicate flasks of each combination were performed (12 flasks per nitrogen source).

Samples preparation for antimicrobial and antioxidant activities assays

The dried mycelium (~1 g) obtained after different growth times (15, 30, 45 and 60 days) and in the presence of each nitrogen source (potassium nitrate, ammonium nitrate, biammonium phosphate and sodium nitrite) was extracted by stirring with 100 mL of methanol at 25 ºC at 150 rpm for 24h and filtered through Whatman nº 4 paper. The residue was then extracted with one additional 100 mL portion of methanol, as described earlier. The combined methanolic extracts were evaporated at 40 ºC to dryness and redissolved in methanol for antioxidant activity assays, or in DMSO for antimicrobial activity assays, at a concentration of 10 mg/mL, and stored at 4 ºC for further use.

Antimicrobial activity

Standards and reagents. Ampicillin and cycloheximide were of the highest available quality, and purchased from Merck (Darmstadt, Germany). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

Microorganisms and culture conditions. Microorganisms CECT were obtained from the Spanish type culture collection (CECT) of Valencia University, while microorganisms ESA were isolated in the Northeast Hospital Centre (Bragança) from different biological fluids, and deposited in Microbiology Laboratory of *Escola Superior Agrária de Bragança*. Gram + (*Bacillus cereus* CECT 148, *B. subtilis* CECT 498, and *Staphylococus aureus* ESA 7, isolated from pus) and Gram – (*Escherichia coli* CECT 101, *Pseudomonas aeruginosa* CECT 108, and *Klebsiella pneumoniae* ESA 8, isolated from urine) bacteria, and fungi (*Candida albicans* CECT 1394, and *Cryptococcus neoformans* ESA 3, isolated from vaginal fluid) were used to screen mycelia antimicrobial activity. Microorganisms were cultured aerobically at 37 ºC (Scientific

222 model) in nutrient agar medium for bacteria, and at 30 ºC in sabouraud dextrose agar medium for fungi.

Test assays for antimicrobial activity. A screening of antibacterial activities against the Gram - and Gram + bacteria and fungi was performed, following the procedure described in **3.1.4.2** (*Test assays for antimicrobial activity*).

Effect of nitrogen source on antioxidant activity

Standards and reagents. Standards BHA (2-*tert*-butyl-4-methoxyphenol), TBHQ (tert-butylhydroquinone), L-ascorbic acid, α-tocopherol, gallic acid and (+)-catechin were purchase from Sigma Chemical Co. (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals were obtained from Sigma. Methanol was obtained from Pronalab (Lisbon, Portugal). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

Determination of antioxidant components. Phenolics and flavonoids content was determined as described in **3.1.4.2** (*Total bioactive compounds*).

Antioxidant activity assays. DPPH radical-scavenging activity, reducing power, inhibition of erythrocyte hemolysis mediated by peroxyl free radicals and inhibition of lipid peroxidation using the β-carotene linoleate model system were evaluated as described in **3.2.1.2**.

Statistical analysis

All the assays were carried out in triplicate and the results expressed as mean values and standard error or standard deviation (SD). Differences among means were done by analysis of variance (ANOVA), using SAS v. 9.1.3, and averages were compared using Tukey test ($p < 0.05$).

3.3.2.3. Results and discussion

Effect of nitrogen source on mycelia growth rate

The *L. giganteus* mycelium growth (dw) in liquid medium with different nitrogen sources is shown in **Figure 3.3.3**. The mycelium growth did not significantly vary with the nitrogen source used. Diammonium phosphate did not lead to a higher mycelium yield although hydrogenophosphate salts had been referred to as the most suitable nitrogen source for mycelium growth of a majority of mushrooms (Mao and Zhong, 2006).

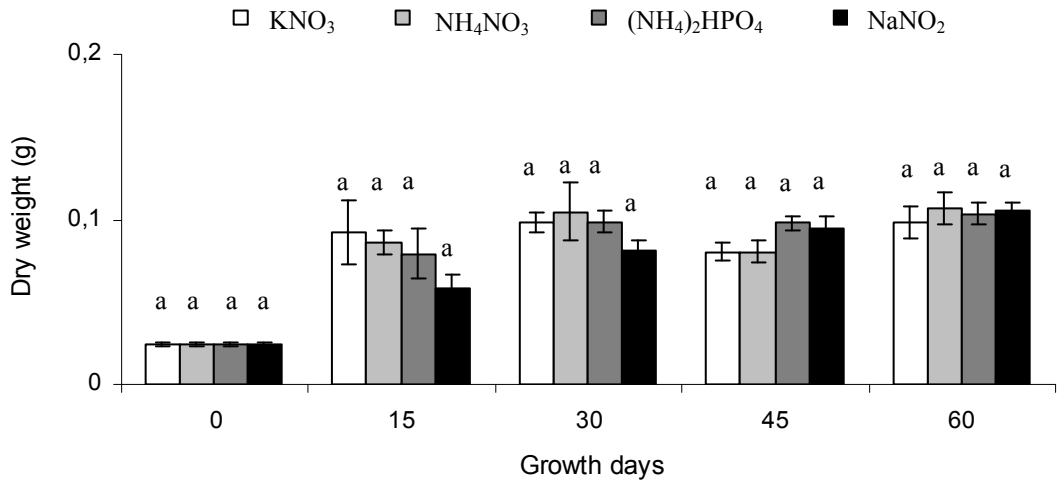


Figure 3.3.3. Mycelium growth (dw) of *Leucopaxillus giganteus* in the presence of different nitrogen sources. In each growth time different letters mean significant differences (p<0.05) .

The mycelium growth in the presence of KNO_3, NH_4NO_3 and $(NH_4)_2HPO_4$ significantly increased until 15 days of incubation maintaining significantly the growth after that time (p<0.05). In the case of $NaNO_2$ the mycelium growth increased significantly until 45 days (0.095 ± 0.0135 g), remaining with a similar dry weight (p>0.05) until 60 days of growth.

Effect of nitrogen source on antimicrobial activity

The mycelia samples were screened for their antimicrobial properties against *B. cereus*, *B. subtilis, S. aureus*, *E. coli*, *P. aeruginosa, K. pneumoniae*, *C. albicans* and *C. neoformans*. The minimal inhibitory concentration (MIC) values for the tested bacteria

and fungi (**Table 3.3.3**) were determined as an evaluation of the antimicrobial activity of the samples.

Despite all the mycelia obtained in the presence of different nitrogen sources revealed antimicrobial activity, the response for each microorganism tested was different. The extracts presented similar antimicrobial capacity, inhibiting only Gram + bacteria and in the order *S. aureus* > *B. cereus* > *B. subtilis*. These results are in agreement with previous studies of our research group on antimicrobial activity of three wild edible mushrooms (*Lactarius deliciosus*, *Sarcodon imbricatu*s and *Tricholoma portentosum*) where the growth of Gram-positive bacteria was well inhibited (see section **3.2.3**). Herein, *S. aureus* was the most susceptible microorganism, presenting MICs of 3.12 and 6.25 mg/mL for the mycelia obtained in the presence of $(NH_4)_2HPO_4$ and for other nitrogen sources, respectively. These results are particularly important considering that *S. aureus* can produce several types of enterotoxins that cause gastroenteritis, which is a major food-borne disease in most countries. Additionally, this species is exceptionally resistant to a number of phytochemicals (Halpin-Dohnalek and Marth, 1989). The tested Gram – bacteria (*E. coli*, *P. aeruginosa* and *K. peumoniae*) and fungi (*C. albicans* and *C. neoformans*) species were resistant to all samples. Diammonium phosphate proved to be the most promissory nitrogen source to produce bioactive compounds that inhibit Gram + bacteria growth, presenting lower MICs and higher growth inhibition zones.

Table 3.3.3. Antimicrobial activity of mycelia extracts obtained using different nitrogen sources after 15 and 60 days of growth.

	MIC (mg/mL)							
Nitrogen source	*B. cereus*	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*E. coli*	*K. peumoniae*	*C. albicans*	*C. neoformans*
KNO_3, 15 days	12.5	25	6.25	25	25	25	25	25
	(+ + +)	(+ +)	(+ + + +)	(-)	(-)	(-)	(-)	(-)
KNO_3, 60 days	12.5	25	6.25	25	25	25	25	25
	(+ + +)	(+ +)	(+ + + +)	(-)	(-)	(-)	(-)	(-)
NH_4NO_3, 15 days	12.5	25	3.13	25	25	25	25	25
	(+ + +)	(+ +)	(+ + + +)	(-)	(-)	(-)	(-)	(-)
NH_4NO_3, 60 days	12.5	25	3.13	25	25	25	25	25
	(+ + +)	(+ +)	(+ + + +)	(-)	(-)	(-)	(-)	(-)
$(NH_4)_2HPO_4$, 15 days	12.5	25	6.25	25	25	25	25	25
	(+ + + +)	(+ +)	(+ + + +)	(-)	(-)	(-)	(-)	(-)
$(NH_4)_2HPO_4$, 60 days	12.5	25	6.25	25	25	25	25	25
	(+ + + +)	(+ +)	(+ + + +)	(-)	(-)	(-)	(-)	(-)
$NaNO_2$, 15 days	25	25	6.25	25	25	25	25	25
	(+)	(+)	(+ + +)	(-)	(-)	(-)	(-)	(-)
$NaNO_2$, 60 days	25	25	6.25	25	25	25	25	25
	(+)	(+)	(+ + +)	(-)	(-)	(-)	(-)	(-)

No antimicrobial activity (-), inhibition zone < 1 mm. Slight antimicrobial activity (+), inhibition zone 2-3 mm. Moderate antimicrobial activity (+ +), inhibition zone 4-5 mm. High antimicrobial activity (+ + +), inhibition zone 6-9 mm. Strong antimicrobial activity (+ + + +), inhibition zone > 9 mm. Standard deviation ± 0.5 mm.

Effect of nitrogen source on antioxidant activity

Table 3.3.4. shows the phenolics and flavonoids concentrations in the mycelium extracts obtained in the presence of four different nitrogen sources: KNO_3, NH_4NO_3, $(NH_4)_2HPO_4$ and $NaNO_2$. Diammonium phosphate proved to be the best nitrogen source for the synthesis of phenolic and flavonoid compounds, showing the highest content at all growth times (23.36 ± 2.05 and 1.35 ± 0.07 mg/g, respectively after 60 days of growth).

Table 3.3.4. Contents of total flavonoids and phenolics (mg/g) of mycelium extracts obtained using different nitrogen sources after 15, 30, 45 and 60 days of growth. In each row different letters mean significant differences ($p<0.05$).

	KNO_3	NH_4NO_3	$(NH_4)_2HPO_4$	$NaNO_2$
		15 days		
Flavonoids	0.69±0.09 a	0.57±0.03 b	0.63±0.11 ba	0.47±0.03 c
Phenolics	6.21±0.18 b	5.5±0.52 b	9.33±0.92 a	3.27±0.41 c
		30 days		
Flavonoids	0.81±0.02 b	1.07±0.06 a	0.88±0.09 b	0.87±0.09 b
Phenolics	8.59±0.5 d	12.07±0.82 c	17.03±0.64 a	16.17± 0.56 b
		45 days		
Flavonoids	1.12±0.02 a	0.74±0.08 b	1.14±0.02 a	1.09±0.19 a
Phenolics	21.91±0.13 b	11.18± 0.23 d	22.71±0.67 a	16.86±1.08 c
		60 days		
Flavonoids	1.01±0.07 d	1.12±0.07 c	1.35±0.07 a	1.23±0.04 b
Phenoicls	19.6±0.66 c	13.71±1.88 d	23.36±2.05 a	21.93±1.46 b

These phenolics amounts were even higher than the values found in the fresh mushroom as already described (see section 3.2.3.). The amounts found in the other extracts (13.71 to 21.93 mg/g) were significantly ($p<0.05$) lower than the content found in the first case. For almost all nitrogen sources, the antioxidant components content increased along the growth time. This is probably a response to the oxidative stress, and therefore free radicals production (Anderson, 1996), related to the mycelium growth.

The highest content of these antioxidant compounds in the mycelium extract grown in the presence of diammonium phosphate might account for the better results found in their antioxidant activity.

As an example of the results obtained **Figure 3.3.4.** presents the scavenging activity on DPPH radicals, reducing power, hemolysis inhibition and LPO inhibition by β-carotene bleaching method of the mycelium extracts after 30 days of growth.

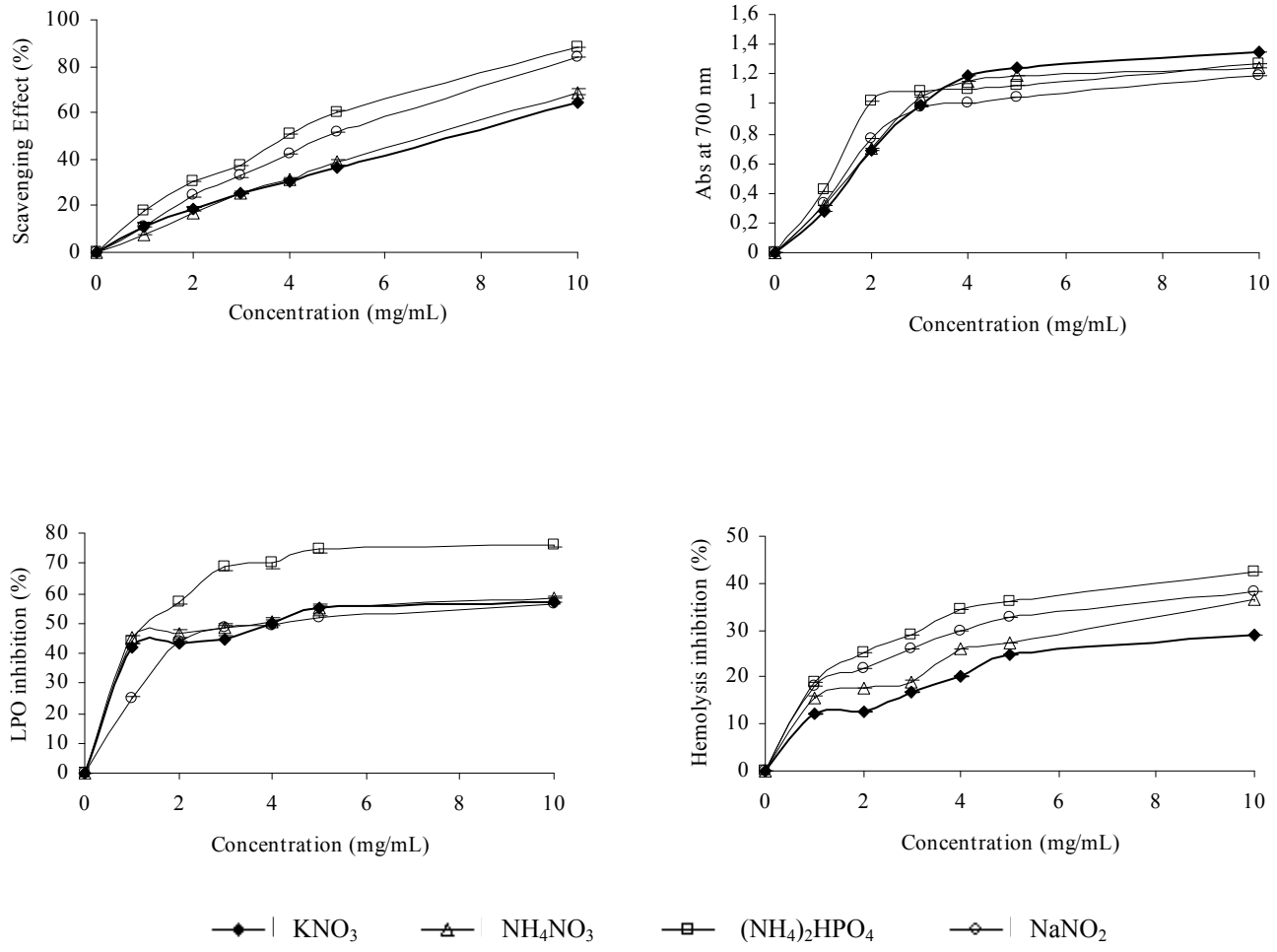


Figure 3.3.4. Antioxidant activity of *Leucopaxillus giganteus* mycelia after 30 days of growth: Scavenging activity on DPPH radicals (%), reducing power, hemolysis inhibition (%) and lipid peroxidation (LPO) inhibition (%).

For an overview of the results, in **Table 3.3.5.** the EC_{50} values for the antioxidant activity assays obtained from each mycelium methanolic extract, after 15, 30, 45, and 60 days of growth are shown. At all the times, mycelia grown in the presence of $(NH_4)_2HPO_4$ revealed better antioxidant properties (significantly lower EC_{50} values; $p<0.05$) than in the other nitrogen sources, which is in agreement with the higher content of phenolics and flavonoids found in the first case.

Table 3.3.5. EC_{50} values obtained in the antioxidant activity assays of mycelium extracts grown in the presence of different nitrogen sources after 15, 30, 45 and 60 days of growth. In each row different letters mean significant differences ($p<0.05$).

EC_{50} (mg/mL)	KNO_3	NH_4NO_3	$(NH_4)_2HPO_4$	$NaNO_2$
		15 days		
Scavenging effect	9.31±0.12 c	9.48±0.09 b	6.29±0.14 d	>10.0 a
Reducing power	1.76±0.001 c	1.98±0.01 b	1.29±0.01 d	3.33±0.02 a
Hemolysis inhibition	>10.0	>10.0	>10.0	>10.0
LPO inhibition	8.79±0.89 a	8.97±0.48 a	5.67±0.47 b	>10.0 a
		30 days		
Scavenging effect	7.48±0.33 a	6.85±0.13 b	3.97±0.04 d	4.79±0.04 c
Reducing power	1.54±0.002 a	1.47±0.01 b	1.14±0.001 d	1.4±0.001 c
Hemolysis inhibition	>10.0	>10.0	>10.0	>10.0
LPO inhibition	4.07±0.02 a	3.73±0.14 ba	1.41±0.42 c	3.32±0.07 b
		45 days		
Scavenging effect	4.14±0.09 c	8.67±0.06 a	2.98±0.02 d	4.46±0.06 b
Reducing power	1.02±0.003 c	1.9±0.004 a	0.9±0.004 d	1.04±0.01 b
Hemolysis inhibition	>10.0	>10.0	>10.0	>10.0
LPO inhibition	1.28±0.45 cb	3.74±0.07 a	1.23±0.44 c	2.1±0.14 b
		60 days		
Scavenging effect	4.33±0.19 b	4.72±0.22 a	2.95±0.07 c	2.85±0.04 c
Reducing power	1.3±0.01 b	1.3±0.01 a	0.99±0.001 c	0.96±0.01 d
Hemolysis inhibition	>10.0	>10.0	>10.0	>10.0
LPO inhibition	1.58±0.2 b	2.6±0.06 a	1.31±0.03 b	1.47±0.1 b

The EC_{50} values obtained for reducing power and LPO inhibition were better than for scavenging effects on DPPH radicals and for hemolysis inhibition mediated by peroxyl free radicals. In the last case, EC_{50} values were always higher than 10 mg/mL.

Significantly negative linear correlations were established between the phenolics and flavonoids contents and EC_{50} values of DPPH scavenging activity (determination coefficient 0.877 for phenolics and 0.678 for flavonoids; $p < 0.001$), reducing power (determination coefficient 0.626 for phenolics and 0.521 for flavonoids; $p < 0.001$) and LPO inhibition (determination coefficient 0.807 for phenolics and 0.669 for flavonoids, $p<0.001$) (**Figure 3.3.5.**). These negative linear correlations prove that the sample with highest phenolics content (diammonium phosphate as nitrogen source) shows higher antioxidant activity and lower EC_{50} values, while the sample with lowest phenolics

content (ammonium nitrate as nitrogen source) presents lower antioxidant activity and higher EC_{50} values.

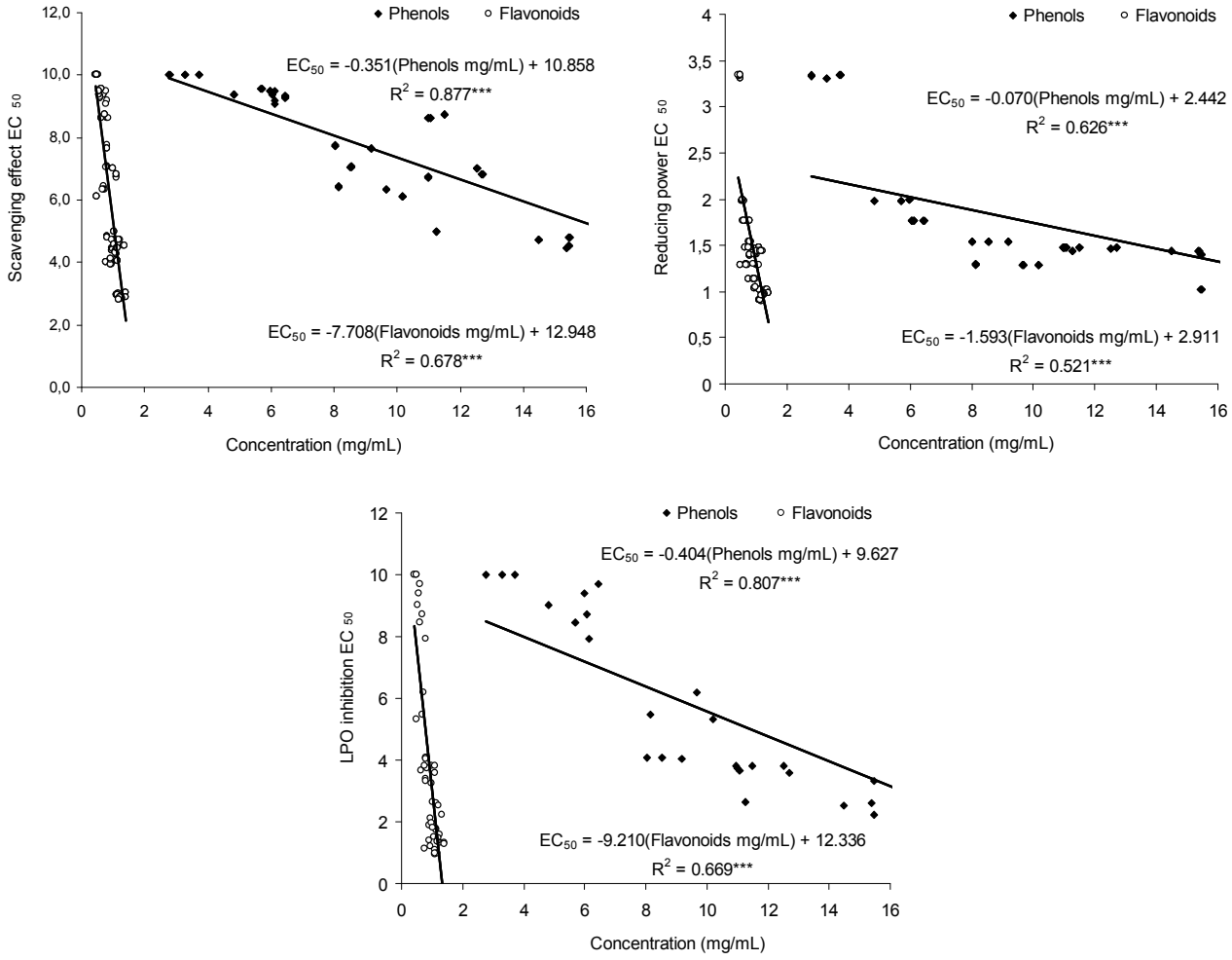


Figure 3.3.5. Correlation established between total phenolics and flavonoids contents and scavenging effect on DPPH radicals, reducing power and LPO inhibition.

In conclusion, the results obtained in this study demonstrate that not only mushrooms but also their mycelia may be good candidates for employment as antimicrobial agents against bacteria responsible for human gastrointestinal and respiratory tract infections. Mushrooms mycelia may also constitute a good source of healthy compounds, namely phenolics, suggesting that they could be useful in the prevention of diseases in which free radicals are implicated. To our best knowledge, the present study was the first report to demonstrate that the bioactive properties (antimicrobial and antioxidant activities), and nutraceuticals production of mushroom mycelia depends on the nitrogen source used for the mycelium growth.

3.3.3. Phenolics and antioxidant activity of mushroom *Leucopaxillus giganteus* mycelium at different carbon sources

3.3.3.1. Introduction

Reactive oxygen species (ROS) such as hydroxyl and superoxide radicals produced by sunlight, ultraviolet, chemical reactions, and metabolic processes have a wide variety of pathological effects on cellular processes (Aust and Sringen, 1982; Pryor et al., 1982; Torel et al., 1986). Superoxide radical is one of the strongest free radicals in cellular oxidation reactions because, once it forms, it further produces various kinds of cell-damaging free radicals and oxidizing agents (Maccarrone et al., 1997). There are many diseases such as heart disease, cancer, arthritis, and the aging process itself, in which free radicals are implicated. To combat these free radicals the body needs antioxidants (Harman, 1997) and in this respect, flavonoids and other phenolics have been shown to posses an important antioxidant activity towards these radicals, which is principally based on the redox properties of their phenolic hydroxyl groups and the structural relationships between different parts of their chemical structure (Bors and Saran, 1987). Antioxidant compounds can increase shelf life by retarding the process of lipid peroxidation, which is also one of the major reasons for deterioration of food products during processing and storage (Halliwell, 1997; Halliwell & Gutteridge, 1999). Thus a need for identifying sources of antioxidants exists and the search for natural antioxidants, especially of plant origin, has notably increased in recent years (Skerget et al., 2005).

Mushrooms have become attractive as functional foods and sources for the development of drugs and nutraceuticals (Chang, 1996; Chang, 1999), namely for antioxidant compounds (Yen and Hung, 2000; Mau et al., 2002a; Yang et al., 2002; Cheung et al., 2003; Cheung and Cheung, 2005; Lo and Cheung, 2005). In addition to dried mushrooms, alternative or substitute mushroom products are mycelia which could be used as food and food-flavouring material, or in the formulation of nutraceuticals and functional foods.

The nutritional value and taste components of some mushroom mycelia have been studied (Weng, 2003). The growth of an edible mushroom with its fruiting body as the most common edible form is a lengthy and complex process involving the use of solid compost or lignocellulosic waste, such as straw or cotton, followed by a long cultivation

period (Vedder, 1978). Growing mushroom mycelium in liquid culture on a defined nutrient medium has long been a simple and fast alternative method to produce fungal biomass (Cirillo et al., 1960; Litchfield, 1967; Cheung, 1995). The fruiting bodies of edible mushrooms are commonly used in human diets as a source of protein (Gray, 1973) and mycelia have already showncomparable nutritional values to mushroom and fruiting bodies (Hadar and Cohen-Arazi, 1986). Also some mycelia antioxidant properties were described (Mau et al., 2004) but little information is available about the influence of the culture medium on mycelium production and antioxidant activity.

In this study, the influence of four different carbon sources in the production and antioxidant activity of *Leucopaxillus giganteus* (Sowerby) Singer mycelium, a *Basidiomycete* fungus belonging to the order *Agaricales* and family *Tricholomataceae* has been evaluated. *Leucopaxillus* species have been reported to have medicinal activity, being used in chemical industry for extraction of clitocybin antibiotic (Breitenbach and Kränzlin, 1991).

The mycelium growth was followed along the time (15, 30, 45 and 60 days) and the antioxidant activity was evaluated for each carbon source and for each growth day, using several assays. The free radical scavenging capacity and reducing power of the extracts, and also the inhibition of oxidative hemolysis in erythrocytes induced by 2,2'-azobis(2-amidinopropane)dihydrochloride, which has been extensively used as model for the peroxidative damage in biomembrane, were studied. The presence of different antioxidants in the mycelium extracts that can hinder the extent of β-carotene-bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system was also tested. All these antioxidant activity parameters were correlated to the total phenolic and flavonoidic content present in the samples.

3.3.3.2. Materials and methods

Standards and Reagents

Standards BHA (2-*tert*-butyl-4-methoxyphenol), TBHQ (tert-butylhydroquinone), L-ascorbic acid, α-tocopherol, gallic acid and (+)-catechin were purchase from Sigma (St. Louis, MO, USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Folin and Ciocalteu's reagent is from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma Chemical Co. (St. Louis,

MO, USA). Methanol was obtained from Pronalab (Lisbon, Portugal). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

Samples

Mycelia of *L. giganteus* (Sowerby) Singer was isolated from sporocarps collected under grassland in Bragança (Northeast of Portugal) (Herbarium of Agrarian School - *Instituto Politécnico Bragança*) on solid Melin-Norkans (MMN) medium pH 6.6 (NaCl 0.025 g/L; $(NH_4)_2HPO_4$ 0.25 g/L; KH_2PO_4 0.50 g/L; $FeCl_3$ 0.050 g/L; $CaCl_2$ 0.50 g/L; $MgSO_4.7H_2O$ 0.15 g/L; thiamine 0.10 g/L; casaminoacid 1.0 g/L; malt extract 10 g/L; glucose 10 g/L; agar 20 g/L), following Brundrett et al. (1996). The strain was maintained in the same medium at 25ºC in the dark and sub-cultured every month.

Effect of carbon source on mycelia growth rate

For aseptic establishment of assay, 10 hyphal plugs (5 mm diameter) of 1-week-old *L. giganteus* mycelia were transferred into flasks (700 mL) containing 250 mL of MMN liquid medium performed with four different carbon sources: glucose, sucrose, fructose and mannitol. Inoculated flasks were shaken and maintained in the dark at 25ºC. After 15, 30, 45 and 60 days of growth the mycelium was recovered from the liquid medium by filtration, washed with distilled water, weighted (fw) and dried at 50ºC, during 24h, to obtain the yield of biomass (dw). Three replicate flasks of each combination were performed (12 flasks per carbon source).

Effect of carbon source on antioxidant activity

The dried mycelium (~1 g) obtained after different growth times (15, 30, 45 and 60 days) and in the presence of each carbon source (glucose, sucrose, fructose and mannitol) was extracted by stirring with 100 mL of methanol at 25 ºC at 150 rpm for 24h and filtered through Whatman nº 4 paper. The residue was then extracted with one additional 100 mL portion of methanol, as described earlier. The combined methanolic extracts were evaporated at 40 ºC to dryness and redissolved in methanol at a concentration of 10 mg/mL, and stored at 4 ºC for further use.

Determination of antioxidant components

Phenolic compounds and flavonoid contents in the extracts were determined as described in **3.1.4.2** (*Total bioactive compounds*).

Antioxidant activity assays

DPPH radical-scavenging activity, reducing power, inhibition of erythrocyte hemolysis mediated by peroxyl free radicals and inhibition of lipid peroxidation using the β-carotene linoleate model system were evaluated as described in **3.2.1.2**.

Statistical analysis

All the assays were carried out in triplicate and the results are expressed as mean values and standard error or standard deviation. Differences among means were done by analysis of variance (ANOVA), using SAS v. 9.1.3, and averages were compared using Tukey test ($p < 0.05$). A regression analysis, using Exel for Windows Software, was established between phenolic and flavonoid contents and EC_{50} values obtained by different antioxidant assays.

3.3.3.3. Results and discussion

Effect of carbon source on mycelia growth rate

The *L. giganteus* mycelium grown (dw) in liquid medium with different carbon sources is shown in **Figure 3.3.6**.

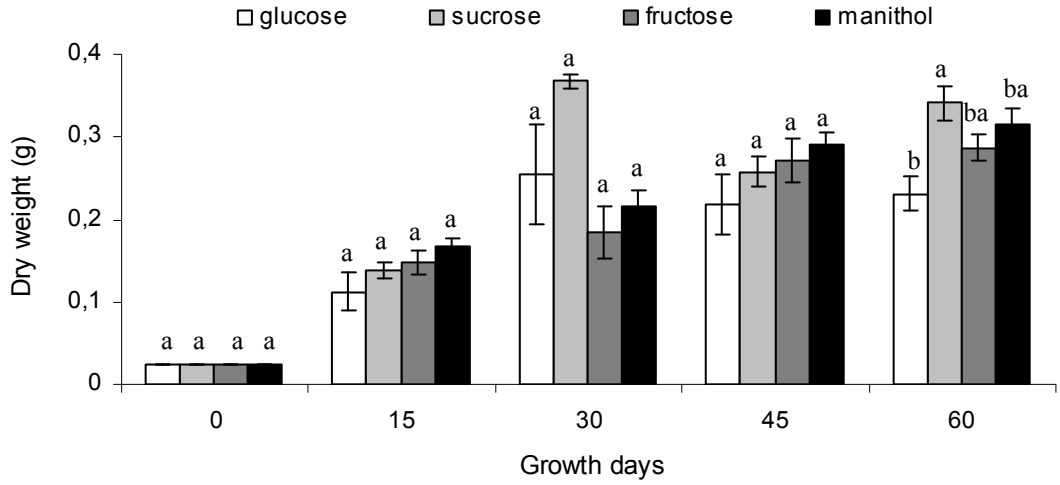


Figure 3.3.6. Mycelium growth (dw) of *Leucopaxillus giganteus* in the presence of different carbon sources. Each value is expressed as mean ± standard error (n=3). In each growth time different letters mean significant differences ($p<0.05$).

The mycelium growth varied with the sugar, being the disaccharide sucrose the best carbon source. Glucose led to a lower mycelium yield although it has been referred to as the most suitable carbon source for mycelium growth of a majority of mushrooms (Chang, 1989; Wang, 1993; Yang, 1996). The mycelium growth in the presence of sucrose significantly increased until 30 days of incubation (0.367 ± 0.0175 g) decreasing after 45 days of growth (p<0.05). Although in the presence of glucose the mycelium growth increased significantly until 30 days (0.255 ± 0.1215 g), the dry weight obtained after 45 and 60 days did not vary significantly (p>0.05). Using fructose as carbon source the mycelium yield significantly increased from 0.024 ± 0.0019 g to 0.270 ± 0.0531 (after 45 days), remaining with a similar dry weight (p>0.05) until 60 days of growth. The presence of mannitol significantly increased the mycelium growth along the incubation time (p<0.05).

The maximum yield of the mycelium for sucrose (disaccharide) and glucose (aldohexose) was obtained after 30 days of incubation, while for fructose (ketohexose) and mannitol (hydroxylated monosaccharide) was obtained only after 60 days. This result implies that the mycelium growth not only varies with the carbohydrate source, but also changes with the time period of growth.

Effect of carbon source on antioxidant activity

Table 3.3.6 shows the phenolics and flavonoids concentrations in the mycelium extracts obtained in the presence of the four different carbon sources (glucose, sucrose, fructose, mannitol). Even though sucrose was more efficient for the mycelium growth, glucose proved to be the best carbon source for the synthesis of phenolics and flavonoids compounds, showing the highest content al all growth times (12.7 ± 1.09 and 1.9 ± 0.55 mg/g, respectively after 60 days of growth). These phenolics amounts were even higher than the value found in the fresh mushroom as we had already described in a previous study (see chapter **3.2.1.**). The amount found in the other extracts (2.9 to 5.7 mg/g) was significantly (p<0.05) lower than the content found in the first case. In general, monosaccharides (glucose, fructose) seemed to be better than disaccharides (sucrose) to the antioxidants synthesis. In fact, the presence of monosaccharides significantly increased (p<0.05) the phenolics content along the time. Mannitol (hydroxylated monosaccharide) led to lower phenolics and flavonoids concentrations (2.9 ± 0.94 and 1.0 ± 0.13 mg/g, respectively).

Table 3.3.6. Flavonoids and phenolics (mg/g)[a] contents in the mycelium extracts obtained using different carbon sources (glucose, sucrose, fructose and mannitol) after 15, 30, 45 and 60 days of growth.

	Glucose	Sucrose	Fructose	Mannitol
		15 days		
Flavonoids	1.3±0.29 a	0.8±0.08 b	1.0±0.11 ab	0.5±0.09 b
Phenolics	6.9±0.71 a	2.1±0.71 b	2.8±0.62 b	2.3±0.71 b
		30 days		
Flavonoids	1.4±0.26 a	0.8±0.05 bc	1.1±0.18 ab	0.7±0.11 c
Phenolics	8.0±0.83 a	2.3±0.70 c	4.6±0.90 b	2.2±0.68 c
		45 days		
Flavonoids	1.7±0.57 a	1.0±0.07 ab	1.1±0.12 ab	0.9±0.11 b
Phenolics	9.6±1.10 a	2.6±1.21 bc	5.2±0.69 b	2.5±0.89 c
		60 days		
Flavonoids	1.9±0.55 a	1.0±0.17 b	1.4±0.11 ab	1.0±0.13 b
Phenolics	12.7±1.09 a	3.5±0.94 b	5.7±0.97 b	2.9±0.94 b

[a] Each value is expressed as mean ± standard deviation (n=3). In each row different letters mean significant differences ($p<0.05$).

For all the carbon sources, the antioxidant components content increased along the growth time (15 to 60 days). This is probably a response to the oxidative stress, and therefore free radicals production (Anderson, 1996), related to the mycelium growth.

Polyphenols include other subclasses besides flavonoids, such as phenolic acids, stilbenes, lignans, tannins, and oxidized polyphenols. Many of these compounds display a large diversity of structures and escape quantification, usually carried out by HPLC (High Performance Liquid Chromatography) and diode array detection, because (i) there is lack of commercial standards and (ii) numerous structures are not yet elucidated (Georgé et al., 2005). The method of Folin-Ciocalteu is, therefore, largely used to evaluate total phenols despite all the interferences present in this method since the reagent (mixture of phosphotungstic acid and phosphomolibdic acid) reacts with other no phenolic reducing compounds leading to an overvaluation of the phenolic content. It is known that ascorbic acid is the main reducing agent, which can interfere in the Folin-Ciocalteu reaction (Georgé et al., 2005), but ascorbic acid content in *Leucopaxillus giganteus* is very low as we reported in a previous work (chapter **3.2.1**). In this study, the assays were performed in the whole extract, since it could be more beneficial than isolated constituents due to additive and synergistic effects; also, a bioactive individual

component can change its properties in the presence of other compounds present in the extracts (Liu, 2003).

The highest content of these antioxidant compounds in the mycelium extract grown in the presence of glucose might account for the better results found in their antioxidant activity (Velioglu et al., 1998; Ferreira et al., 2007).

Figure 3.3.7 shows the scavenging activity on DPPH radicals, reducing power, hemolysis inhibition and antioxidant activity by β-carotene bleaching method of the mycelium extracts after 30 days of growth.

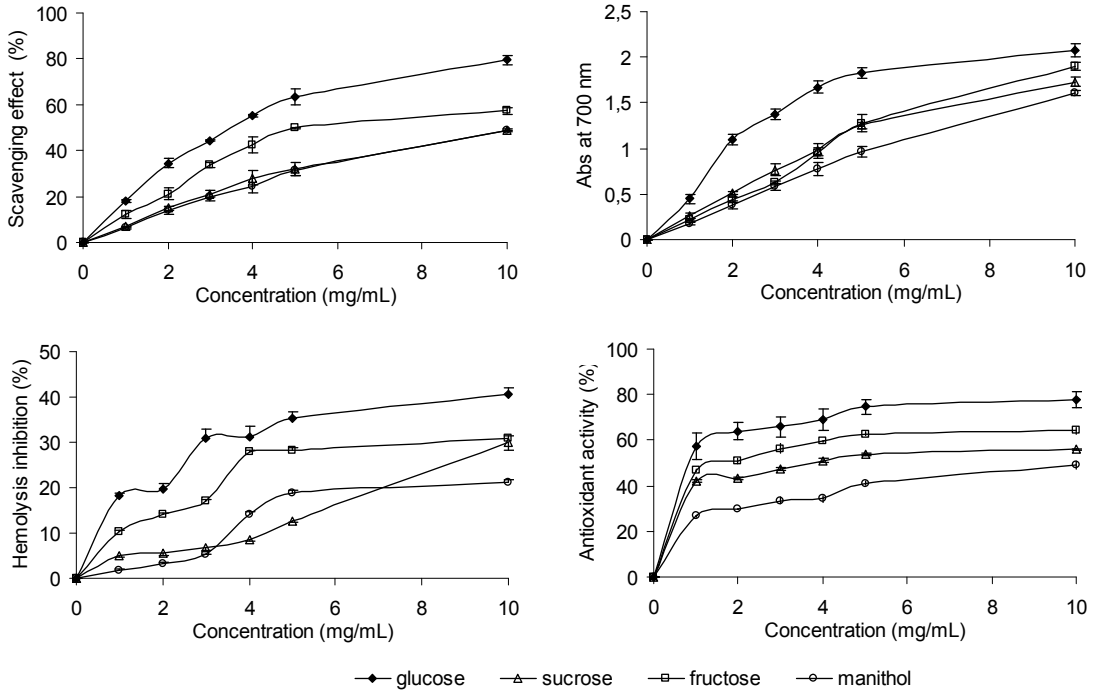


Figure 3.3.7. Scavenging activity on DPPH radicals (%), reducing power, hemolysis inhibition (%) and antioxidant activity by β-carotene bleaching method (%) of the mycelium extracts after 30 days of growth. Each value is expressed as mean ± standard error (n=3).

The radical scavenging activity (RSA) values of mycelium methanolic extracts were expressed as the ratio percentage of sample absorbance decrease and the absorbance of DPPH solution in the absence of extract at 517 nm. From the analysis of **Figure 3.3.7**, it can be concluded that the scavenging effects of mycelium extracts on DPPH radicals increased with the concentration increase and were very good for the extract from the glucose-medium (79.5% at 10 mg/mL), but lower than the scavenging effects of BHA (96% at 3.6mg/mL) and α-tocopherol (95% at 8.6mg/mL). The RSA values at 10

mg/mL were moderate for the other carbon sources (fructose- 57.4%, sucrose- 49.1%, mannitol- 48.7%). The extracts from the sucrose and mannitol medium showed a very similar scavenging activity, after 30 days of growth.

The reducing power of the mycelium extracts increased with concentration. Reducing powers obtained for all the extracts were excellent (**Figure 3.3.7**); at 10 mg/mL were higher than 1.60 and in the order of glucose>fructose>sucrose>mannitol. Reducing power of BHA at 3.6 mg/mL and α-tocopherol at 8.6 mg/mL was only 0.12 and 0.13, respectively. Methanolic extracts obtained in the presence of sucrose and fructose showed similar reducing power values. It was reported that the reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Shimada et al., 1992). Accordingly, the extract from the glucose-medium might contribute for the synthesis of higher amounts of reductone, which could react with free radicals to stabilize and block radical chain reactions.

In this study, the protective effect of the mycelium extracts on hemolysis by peroxyl radical scavenging activity was also investigated. AAPH is a peroxyl radical initiator that generates free radicals by its thermal decomposition and will attack the erythrocytes to induce the chain oxidation of lipid and protein, disturbing the membrane organization and eventually leading to hemolysis. The mycelium extracts inhibited hemolysis, as a result of protection against the oxidative damage of cell membranes of erythrocytes from ram induced by AAPH, in a concentration-dependent manner (**Figure 3.3.7**). Once more, the extract from the glucose-medium showed higher protective effect against erythrocytes hemolysis (40.7% at 10 mg/mL) than the other carbon sources (fructose- 30.8%, sucrose- 29.9%, mannitol- 21.1%). However, the inhibition percentage of the standard L-ascorbic acid on hemolysis of red blood cells was much higher (94.6% at 1 mg/mL) than those of mycelium extracts.

The antioxidant activity of the mycelium extracts measured by the bleaching of β-carotene is presented in **Figure 3.3.7**. The linoleic acid free radical attacks the highly unsaturated β-carotene models. The presence of different antioxidants can hinder the extent of β-carotene-bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system (Jayaprakasha et al., 2001). Accordingly, the absorbance decreased rapidly in samples without antioxidant, whereas in the presence of an antioxidant, they retained their colour, and thus absorbance, for a longer time.

Antioxidant activity of mycelium extracts growth in the presence of glucose, fructose, glucose and mannitol increased with their increasing concentration. Their antioxidant activities were 77.7%, 64.1%, 55.9% and 49.1% at 10 mg/mL, but antioxidant activity of TBHQ standard reached 82.2% at 2 mg/mL. It is probable that the antioxidative components in the mycelium extracts can reduce the extent of β-carotene destruction by neutralizing the linoleate free radical and other free radicals formed in the system. Again, mycelia grown in the presence of glucose were the most effective for antioxidant activity.

In **Table 3.3.7** the EC_{50} values for the antioxidant activity assays obtained from each mycelium methanolic extract, after 15, 30, 45, and 60 days of growth are presented. At all the growth times, the extract from the glucose-medium revealed better antioxidant properties (significantly lower EC_{50} values; $p<0.05$) than the other carbon sources, which is in agreement with the higher content of phenolics found in the first case. The EC_{50} values obtained for reducing power and antioxidant activity using the linoleate-β-carotene system were better than for scavenging effects on DPPH radicals and for hemolysis inhibition mediated by peroxyl free radicals. In the last case, EC_{50} values were always higher than 10 mg/mL. EC_{50} values for all the antioxidant activity assays decreased along the growth time.

Table 3.3.7. Scavenging effect, reducing power and antioxidant activity EC_{50} values[a] of mycelium extracts grown in the presence of different carbon sources (glucose, sucrose, fructose and mannitol) after 15, 30, 45 and 60 days of growth.

EC_{50} (mg/mL)	Glucose	Sucrose	Fructose	Mannitol
		15 days		
Scavenging effect	3.6±0.121 c	>10.0 a	6.2±0.040 b	>10.0 a
Reducing power	1.7±0.253 c	2.3±0.129 ab	2.1±0.098 bc	2.9±0.320 a
Antioxidant activity	1.5±0.097 c	>10.0 a	9.8±0.057 b	>10.0 a
		30 days		
Scavenging effect	3.5±0.031 c	>10.0 a	5.1±0.160 b	>10.0 a
Reducing power	1.1±0.154 c	2.3±0.166 ab	2.0±0.195 b	2.6±0.361 a
Antioxidant activity	0.9±0.153 d	3.7±0.401 b	1.8±0.342 c	>10.0 a
		45 days		
Scavenging effect	3.4±0.126 c	>10.0 a	4.7±0.176 b	>10.0 a
Reducing power	1.0±0.086 b	2.1±0.273 a	1.8±0.265 a	2.5±0.387 a
Antioxidant activity	0.8±0.087 c	2.3±0.212 b	0.9±0.132 c	4.5±0.542 a

		60 days		
Scavenging effect	3.2±0.155 c	>10.0 a	4.3±0.203 b	>10.0 a
Reducing power	0.8±0.133 c	1.9±0.140 a	1.5±0.197 b	2.3±0.187 a
Antioxidant activity	0.7±0.126 b	1.0±0.129 b	0.9±0.117 b	1.6±0.238 a

[a] Each value is expressed as mean ± standard deviation (n=3). In each row different letters mean significant differences (p<0.05).

Significantly negative linear correlations (**Figure 3.3.8**) were established between the phenolics and flavonoids contents and EC_{50} values of DPPH scavenging activity (determination coefficient 0.664 for phenolics and 0.519 for flavonoids; $p < 0.001$), reducing power (determination coefficient 0.738 for phenolics and 0.588 for flavonoids; $p < 0.001$) and antioxidant activity (determination coefficient 0.329 for phenolics, p<0.001 and 0.361 for flavonoids, p<0.001).

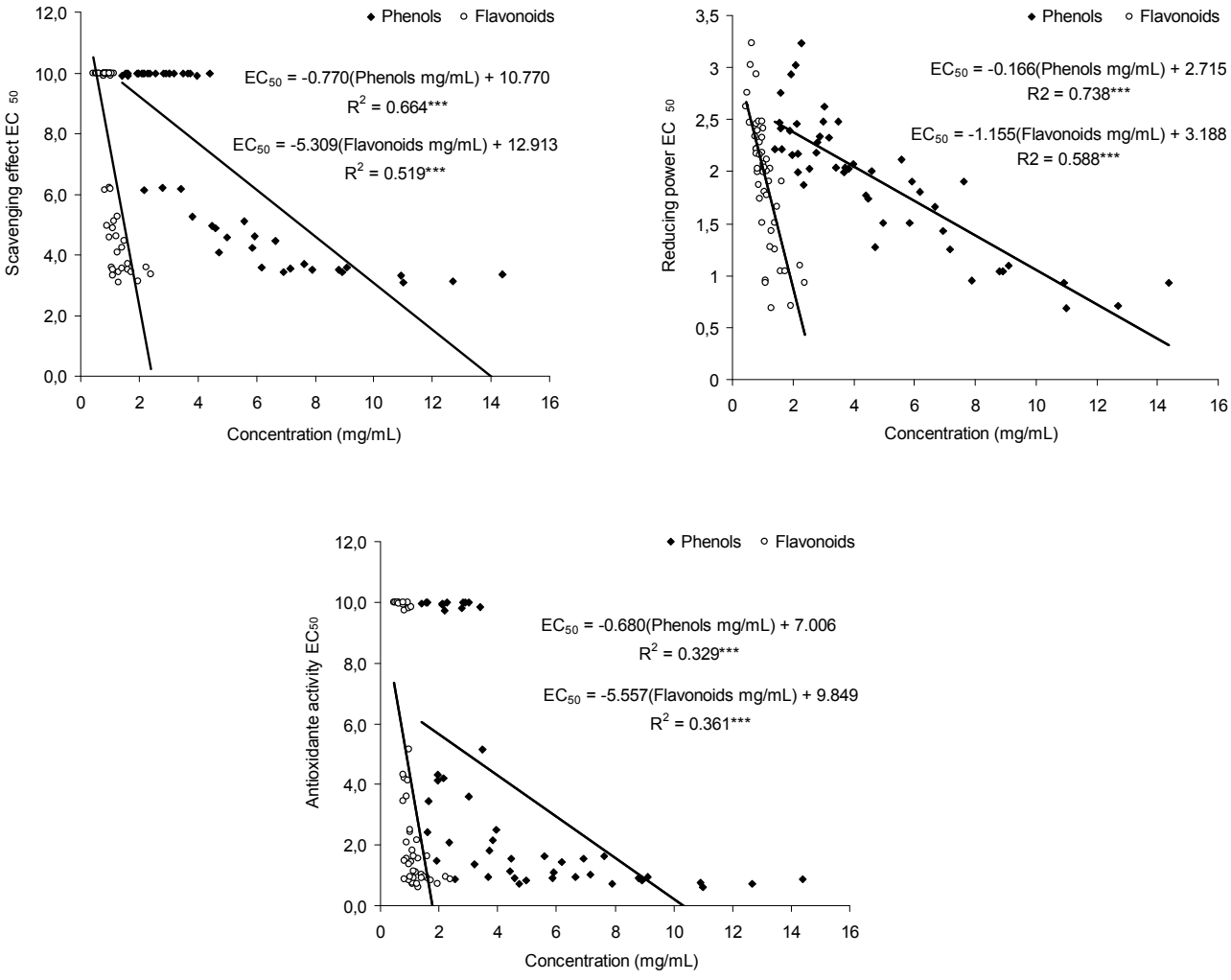


Figure 3.3.8. Correlation established between phenolics and flavonoids contents and scavenging effect on DPPH radicals, reducing power and antioxidant activity.

In conclusion, the negative linear correlations obtained in this study prove that the sample with highest phenolics content shows higher antioxidant activity and lower EC_{50} values (glucose as carbon source), while the sample with lowest phenolics content presents lower antioxidant activity and higher EC_{50} values (mannitol as carbon source). The correlations also support that the mechanism of action of the extracts for the antioxidant activity may be identical, being related with the content in phenolics and flavonoid compounds, and their free radical scavenging activity. To our best knowledge, the present study is the first report to demonstrate that the concentrations of antioxidative components in the extracts depend on the carbon source used for the mycelium growth, increasing along the growth time as a response to the oxidative stress and therefore free radicals production.

3.4. Influence of conservation treatment, cooking and fruiting body maturity in the chemical composition and bioactive properties of Portuguese wild mushrooms

3.4.1 Effects of conservation treatment and cooking on the chemical composition and antioxidant activity of Portuguese wild edible mushrooms

3.4.1.1 Introduction

Wild mushrooms are becoming more and more important in our diet for their nutritional (Crisan and Sands, 1978; Breene, 1990; Manzi et al., 1999) and pharmacological (Bobek et al., 1991; Bobek et al., 1995; Bobek et al., 1999) characteristics. The high protein and low fat/energy contents of wild edible mushrooms, reported by many workers (Aletor, 1995; Fasidi, 1996; Longvah and Deosthale, 1998; Yildiz et al., 1998) and also observed in this work (see chapter 3.1.1), make them excellent foods for use in low caloric diets. Concerning the pharmacological potential such as antimicrobial (Díez and Alvarez, 2001; Agahar-Murugkar and Subbulakshmi, 2005), antiviral, antitumor, antiallergic, immunomodulating, anti-inflammatory, antiatherogenic, hypoglycemic, hepatoprotective properties (Lindquist et al., 2005), mushrooms have also become attractive as a functional food and as sources for the development of drugs and nutraceuticals. Among them, phenolic compounds exhibit potent antioxidant activities (Yen and Hung, 2000; Yang et al., 2002; Mau et al., 2004; Cheung and Cheung, 2005; Ribeiro et al., 2006; Ferreira et al., 2007). Antioxidants can scavenge free radicals and increase shelf life by retarding the process of lipid peroxidation, and the consumption of antioxidants-rich foods could bring diverse physiological benefits to the consumer, such as protection against human diseases associated with oxidative stress, like coronary heart disease and cancer (Halliwell and Gutteridge, 1999).

Nevertheless, edible mushrooms are characterized by a short shelf life (1-3 days at room temperature), linked to the occurrence of post-harvest changes. These changes are due to the high moisture content of the carpophores and to the high activity of enzymes such as proteases or polyphenol oxidases, responsible for protein and sugar decrease and for browning reactions during storage (Czapski and Szudyga, 2000). The drying and deep-

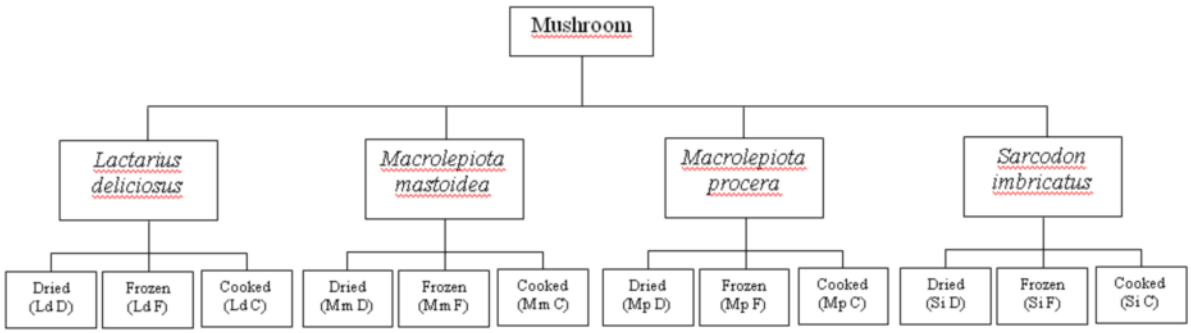
freezing processes have been used to increase storage stability and facilitate mushroom consumption without seasonal constraints (Manzi et al., 2004). Chemical and nutritional characteristics of mushrooms are closely linked not only to species but also to processing (Longvah and Deosthale, 1998; Manzi et al., 1999; Díez and Alvarez, 2001) and cooking practices (Manzi et al., 2001; Manzi et al., 2004; Dikeman et al., 2005). Despite this evidence, there are no detailed studies on the influence in fatty acid and sugars profiles. There are also reports about the changes in the content of health-promoting compounds and antioxidant activity of several vegetables after cooking, such as broccoli (Gliszczynska-Swiglo et al., 2006), cabbages (Ismail and Lee, 2004), amaranth, cowpea, peanut, pumpkin and sweet potato leaves (Mosha et al., 1997), but there are no reports in mushrooms concerning this aspect.

The objective of this study was to evaluate the modifications induced by different conservation treatments (drying and freezing) or cooking in the chemical composition and antioxidant properties of four Portuguese wild edible mushroom species (*Lactarius deliciosus*, *Macrolepiota mastoidea*, *Macrolepiota procera*, *Sarcodon imbricatus*).

3.4.1.2. Materials and methods

Samples

Samples of *Lactarius deliciosus* (L.) Gray, *Sarcodon imbricatus* (L.) P. Karst. *Macrolepiota mastoidea* (Fr.) Singer and *Macrolepiota procera* (Scop.) Singer, were collected under live pine trees (*Pinus* sp.) for the first two species and under oak trees *(Quercus pyrenaica* Willd.) for the lasts species, in Bragança (Northeast of Portugal), in autumn 2006. Taxonomic identification was made according to several authors (Moser, 1983; Courtecuisse and Duhem, 1995) and representative voucher specimens were deposited at the herbarium of *Escola Superior Agrária* of *Instituto Politécnico de Bragança*. After collection and taxonomic identification, all the mushrooms were immediately submitted to different treatments, according to this chart:



Drying was performed in an oven at 40 ºC; Freezing was carried out at -20 ºC. Mushrooms were cooked with olive oil (extra-virgin), salt and onion; this procedure corresponds to the traditional cooking practice used for these Portuguese mushroom species. After cooking, the onion and excess olive oil were removed.
All the samples were lyophilised (Ly-8-FM-ULE, Snijders, Holland) and submitted to chemical composition and antioxidant activity analysis.

Standards and reagents

All reagents were of analytical grade purity: methanol and diethyl ether were supplied by Lab-Scan (Lisbon, Portugal); toluene from Riedel-de-Haën (Hanover, Germany); sulphuric acid from Fluka. The fatty acids methyl ester (FAME) reference standard mixture 37 (fatty acids C4 to C24; standard 47885-U) was from Supelco (Bellefonte, PA, USA) and also purchased from Sigma Chemical Co. (St. Louis, MO, USA), as well as other individual fatty acid isomers and the standards used in the antioxidant activity assays: BHA (2-*tert*-butyl-4-methoxyphenol), TBHQ (tert-butylhydroquinone), L-ascorbic acid, α-tocopherol, gallic acid and (+)-catechin. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Folin and Ciocalteu's reagent is from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

Chemical composition

Samples of mushrooms were analysed for chemical composition (moisture, protein, fat, carbohydrates and ash) as previously described in **3.1.1.2** (C*hemical composition*).

Fatty acid composition

Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GLC-FID)/capillary column as described in **3.1.4.2.** (*Fatty acid composition*).

Sugar composition

Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) as described in **3.1.1.2** (*Sugar composition*).

Antioxidant activity

Sample preparation. The samples (~3 g) were extracted by stirring with 100 mL of methanol at 25 ºC at 150 rpm for 24h and filtered through Whatman nº 4 paper. The residue was then extracted with two additional 100 mL portions of methanol, as described earlier. The combined methanolic extracts were evaporated at 40 ºC to dryness and redissolved in methanol at a concentration of 50 mg/mL, and stored at 4 ºC for further use.

Determination of phenolic compounds. Phenolics and flavonoids content was determined as described in **3.1.4.2** (*Total bioactive compounds*).

DPPH radical-scavenging activity, reducing power and *inhibition of lipid peroxidation using the β-carotene linoleate model system* were evaluated as described in **3.2.1.2**. *Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)* was determined as described in **3.2.2.2**.

Statistical analysis

For each one of the mushroom species three samples were analysed and also all the assays were carried out in triplicate. The results were expressed as mean values and standard error (SE) or standard deviation (SD). The effects of conservation treatments (drying, freezing) and cooking on the mushrooms chemical composition and antioxidant activity were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SAS v. 9.1.3 program.

3.4.1.3. Results and discussion

Effects of conservation and cooking on the chemical composition

The results of the chemical composition and estimated energetic value obtained for the wild mushroom species are shown in **Table 3.4.1**. The moisture ranged from 64.36 g/100 g of fresh weight in cooked *M. procera* and 93.89 g/100 g in dried *S. imbricatus*. The dry matter content of raw mushrooms was higher as compared to cooked mushrooms, but no significant differences were obtained between dried and frozen samples. Carbohydrates were the predominant macronutrients and ranged from 16.40

g/100 g in cooked *M. procera* and 80.38 g/100 g in the corresponding dried sample. Protein was the second component most abundant and varied between 7.62 g/100 g in dried *M. procera* and 29.98 g/100 g in *S. imbricatus* dried sample. Fat ranged from 1.45 g/100 g in dried *M. procera* and 63.03 g/100 g in cooked *M. mastoidea*. The high protein and carbohydrate and low fat characteristic of the edible wild mushrooms have been previously reported by other workers (Aletor, 1995; Longvah and Deosthale, 1998; Díez and Alvarez, 2001). Ash content varied between 3.54 g/100 g in cooked *M. mastoidea* and 14.28 g/100 g in dried *L. deliciosus*. Reducing sugars are only a small part of carbohydrates content since polysaccharides such as chitin and starch are the most abundant mushrooms carbohydrates. The heat inherent to the cooking process could lead to polysaccharides hydrolysis and subsequent sugars release; accordingly cooked samples present higher amounts of reducing sugars.

Table 3.4.1. Moisture (g/100 g of fresh weight), proximate chemical composition (g/100 g of dry weight) and energetic value (Kcal/100 g of dry weight) of the wild edible mushrooms (mean ± SD; n=3). In each row and for each species, different letters mean significant differences ($p<0.05$).

Samples	Moisture	Total fat	Crude protein	Ash	Carbohydrates	Reducing sugars	Energy
L. deliciosus D	90.05 ± 1.84 a	6.47 ± 0.70 b	17.87 ± 1.62 c	14.28 ± 0.22 a	60.30 ± 2.73 a	0.48 ± 0.11 b	370.90 ± 3.97 c
L. deliciosus F	88.25 ± 3.56 a	8.45 ± 3.49 b	24.33 ± 1.81 b	9.53 ± 1.60 b	57.68 ± 2.97 a	0.54 ± 0.01 ba	404.17 ± 17.94 b
L. deliciosus C	65.74 ± 0.77 b	35.95 ± 2.74 a	29.64 ± 1.96 a	4.53 ± 0.08 c	28.77 ± 3.91 b	0.66 ± 0.02 a	561.63 ± 13.43 a
M. mastoidea D	88.69 ± 3.69 a	2.55 ± 0.13 c	21.89 ± 2.31 b	7.96 ± 0.30 b	67.60 ± 1.43 a	0.30 ± 0.02 c	380.89 ± 1.77 b
M. mastoidea F	89.80 ± 0.8 a	3.05 ± 0.17 b	24.51 ± 3.97 a	11.76 ± 0.49 a	60.68 ± 1.26 b	0.46 ± 0.04 b	368.22 ± 3.98 c
M. mastoidea C	70.30 ± 1.87 b	63.03 ± 0.02 a	10.10 ± 0.46 c	3.54 ± 0.58 c	23.38 ± 0.44 c	0.67 ± 0.04 a	700.96 ± 2.21 a
M. procera D	90.01 ± 1.73 a	1.45 ± 0.13 b	7.62 ± 0.08 c	9.86 ± 0.72 b	80.38 ± 0.19 a	0.32 ± 0.01 c	365.01 ± 0.59 b
M. procera F	89.85 ± 1.85 a	2.18 ± 0.07 b	9.36 ± 0.91 b	9.16 ± 0.59 c	79.28 ± 1.41 a	0.56 ± 0.04 b	379.87 ± 6.05 b
M. procera C	64.36 ± 7.88 b	57.43 ± 2.93 a	11.85 ± 0.13 a	5.73 ± 0.09 a	16.40 ± 2.68 b	0.79 ± 0.02 a	623.67 ± 11.14 a
S. imbricatus D	93.89 ± 5.20 a	3.45 ± 0.18 c	29.98 ± 0.30 a	12.14 ± 0.32 a	54.43 ± 0.76 b	0.49 ± 0.01 a	368.69 ± 1.04 c
S. imbricatus F	92.95 ± 1.23 a	8.94 ± 2.99 b	25.71 ± 4.23 a	8.31 ± 0.31 b	55.98 ± 3.45 b	0.46 ± 0.02 a	404.01 ± 6.48 b
S. imbricatus C	68.54 ± 1.78 b	16.21 ± 1.15 a	15.02 ± 0.33 b	4.82 ± 0.32 c	63.95 ± 1.50 a	0.48 ± 0.04 a	461.74 ± 6.05 a

D- dried; F-frozen; C- cooked

Regarding results in a fresh-basis, cooking procedures significantly increased nutrient concentration by decreasing the water content; nevertheless, if values are calculated on a dried-basis (**Table 3.4.1**), a significant carbohydrates and protein loss can be observed. These results are in agreement with other studies on different mushroom species. Manzi *et al.* (2001, 2004) reported that cooking processes result in a loss of moisture and a subsequent concentration of nutrients, but also that cooking may promote a loss of nutrients due to interactions among constituents, chemical reactions, solubility in cooking medium, and (or) thermal degradation. The same authors (Manzi et al., 2004) described a significant decrease for dry matter, fat, protein and carbohydrates after cooking (70-72%) in dried samples and a concentration increase (90-94%) in fresh samples of *Agaricus bisporus*, *Pleurotus ostreatus* and *Boletus sp.* Dikeman *et al.* (2005) also reported cooking losses and, therefore, concentration of dry matter constituents in *Agaricus bisporus*, *Grifola frondosa* and *Lentinus edodes*, especially for carbohydrates (starch and total dietary fibber).

The significant higher fat amount found in the cooked mushrooms might be due to the presence of some olive oil used in the cooking procedure, as it can be observed in the fatty acids profile presented below. On the basis of the proximate analysis, it can be calculated that 100 g of the cooked mushrooms assures significantly higher energy values. The highest values are guaranteed by cooked *M. mastoidea* (700.96 Kcal), while dried *M. procera* (365.01 Kcal) give the lowest energy contribution (**Table 3.4.1**). An apparent greater stability of nutrients and subsequent higher energetic contribution was observed in frozen mushrooms, when compared with the more severe drying treatment. Despite all these conclusions, variation in composition among mushrooms may be due to several other factors including mushroom strain/type, composition of growth media, time of harvest, management techniques, handling conditions, and preparation of the substrates (Manzi et al., 2004; Dikeman et al., 2005).

Effects on the fatty acid composition

The results for fatty acid composition, total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) of the studied mushrooms are shown in **Table 3.4.2**.

Table 3.4.2. Fatty acid composition (percent) of the wild edible mushrooms (mean ± SD; n=3).

	Ld D	Ld F	Ld C	Mm D	Mm F	Mm C	Mp D	Mp F	Mp C	Si D	Si F	Si C
C10:0	1.99±0.22	1.82±0.02	0.18±0.01	0.01±0.00	0.01±0.00	*Nd*	0.15±0.03	0.01±0.00	*nd*	0.02±0.00	0.04±0.02	*nd*
C12:0	0.24±0.01	0.35±0.01	0.03±0.00	0.03±0.01	0.03±0.00	*Nd*	0.06±0.01	0.04±0.01	*nd*	0.02±0.00	0.02±0.00	*nd*
C14:0	0.17±0.00	0.14±0.01	0.02±0.00	0.26±0.00	0.21±0.00	0.02±0.00	0.32±0.03	0.20±0.01	0.01±0.00	0.18±0.00	0.14±0.01	0.02±0.00
C15:0	0.28±0.01	0.30±0.01	0.04±0.00	0.18±0.00	0.11±0.00	0.01±0.00	0.35±0.03	0.33±0.02	0.01±0.00	0.87±0.00	0.91±0.00	0.03±0.00
C16:0	6.60±0.11	6.87±0.16	10.19±0.25	16.97±0.12	16.83±0.07	10.49±0.09	19.80±0.26	18.48±0.14	10.30±0.12	13.61±0.08	14.46±0.11	10.60±0.01
C16:1	0.19±0.01	0.26±0.01	0.48±0.01	2.33±0.04	2.82±0.02	0.60±0.01	1.06±0.01	0.72±0.02	0.49±0.00	0.35±0.00	0.42±0.01	0.52±0.00
C17:0	0.15±0.00	0.13±0.00	0.12±0.01	0.11±0.00	0.06±0.00	0.12±0.00	0.21±0.02	0.14±0.01	0.12±0.00	0.08±0.00	0.06±0.00	0.12±0.00
C18:0	44.38±1.34	43.13±0.63	9.32±1.36	1.26±0.04	0.55±0.01	2.83±0.03	2.38±0.06	2.09±0.03	2.81±0.19	3.57±0.13	3.82±0.48	3.27±0.01
C18:1n9c	21.63±0.40	24.41±1.76	64.80±0.42	16.61±0.32	17.36±0.28	71.88±0.11	9.04±0.29	14.46±0.19	73.70±0.34	50.04±0.28	52.40±0.74	72.32±0.34
C18:2n6c	23.19±1.23	21.13±1.33	12.90±1.13	60.59±0.45	60.69±0.34	12.16±0.02	64.55±0.34	62.14±0.26	10.67±0.35	28.59±0.30	25.24±1.16	11.04±0.38
C18:3n3	0.41±0.13	0.50±0.04	0.91±0.02	0.08±0.01	0.11±0.01	0.98±0.00	0.06±0.00	0.11±0.02	0.99±0.01	0.07±0.00	0.08±0.01	1.02±0.01
C20:0	0.18±0.01	0.19±0.00	0.40±0.00	0.12±0.01	0.06±0.00	0.41±0.00	0.16±0.01	0.11±0.01	0.42±0.01	0.92±0.01	1.02±0.02	0.45±0.01
C20:1c	0.03±0.00	0.01±0.00	0.23±0.00	0.10±0.01	0.10±0.00	0.25±0.00	0.06±0.00	0.05±0.00	0.25±0.01	0.07±0.00	0.07±0.00	0.26±0.00
C20:2c	0.04±0.01	0.05±0.01	0.04±0.00	0.08±0.01	0.09±0.00	*Nd*	0.11±0.01	0.06±0.01	0.01±0.00	0.04±0.00	0.05±0.01	*nd*
C22:0	0.16±0.00	0.13±0.04	0.12±0.00	0.12±0.03	0.07±0.01	0.12±0.00	0.24±0.01	0.17±0.00	0.12±0.01	0.59±0.03	0.69±0.02	0.12±0.01
C23:0	0.02 ±0.01	0.32±0.16	0.02±0.00	0.09±0.06	0.04±0.02	0.01±0.00	0.09±0.02	0.10±0.02	0.02±0.01	0.25±0.05	0.07±0.01	0.04±0.01
C24:0	0.20±0.01	0.10±0.09	0.06±0.02	0.75±0.06	0.61±0.01	0.07±0.00	0.89±0.06	0.63±0.02	0.06±0.00	0.58±0.02	0.35±0.03	0.17±0.01
total SFA	54.36±1.72	53.48±1.14	20.51±1.65	19.88±0.33	18.57±0.13	14.08±0.13	24.63±0.55	22.31±0.29	13.88±0.34	20.69±0.34	21.57±0.71	14.82±0.20
total MUFA	21.85±0.40	24.69±1.78	65.51±0.43	19.04±0.37	20.27±0.30	72.73±0.12	10.17±0.31	15.23±0.21	74.44±0.35	50.46±0.29	52.88±0.75	73.09±0.34
total PUFA	23.65±1.36	21.68±1.38	13.84±1.19	60.76±0.47	60.89±0.35	13.14±0.02	64.72±0.35	62.31±0.28	11.67±0.37	28.69±0.30	25.37±1.18	12.06±0.39

nd- not detected. For abbreviation see the chart above.

In general for non-cooking samples, the major fatty acids found were linoleic acid (C18:2; ~60% for *Macrolepiota* species and ~25% for *L. deliciosus* and *S. imbricatus*) and oleic acid (C18:1; ~50% for *S. imbricatus*, ~20% for *L. deliciosus* and ~15% for *Macrolepiota* species), followed by palmitic acid (C16:0; ~ 10% for *L. deliciosus* and ~15% for the other species). For *L. deliciosus* mushroom, stearic acid (C18:0) was also found in significant amounts, being the most abundant fatty acid (~44%). This fact was already observed by us in a previous assay (see chapter 3.1.1.). The prevalence of linoleic (~65%), oleic (~10%) and palmitic (~20%) acids was also observed by Longvah and Deosthale (1998) in the analysis of Indian mushrooms, *Schizophyllum commune* and *Lentinus edodes*. In another study with *Tricholoma* species, oleic (~57%) and linoleic (~28%) acid were also the main fatty acid constituents, whilst other fatty acids detected were found only in small amounts (Díez and Alvarez, 2001). Besides the four main fatty acids already described, thirteen more were identified and quantified (**Table 3.4.2**). There were no differences in the fatty acid profile of dried and frozen samples (**Table 3.4.2**), which is in agreement with the results obtained for total fat presented in **Table 3.4.1**. UFA predominate over SFA in all the mushroom samples (dried, frozen and cooked), with exception for *L. deliciosus* due to the abundance of C18:0 in this case. This is consistent with the observations that, in fresh mushrooms, unsaturated fatty acids predominate over the saturated, in the total fatty acid content (Longvah and Deosthale, 1998; Díez and Alvarez, 2001). Particularly for cooked samples, PUFA content decreased while MUFA percentage increased. Being oleic acid the most abundant fatty acid in olive oil (Perreira et al., 2002), the increase in oleic acid percentage observed in all the cooked samples is probably due to the presence of some olive oil used in mushrooms preparation. SFA slightly decreased in all the samples except in *L. deliciosus*. Considering total MUFA content, cooked *M. procera* had the highest value (74.44%) but contained the lowest PUFA content (11.67%). Otherwise, dried *M. procera* presented the lowest MUFA content (10.17%) and the highest PUFA value (64.72%). In fact, for the cooked samples, it was observed a considerable decrease in linoleic acid contents and, for *L. deliciosus*, also in stearic acid content probably due to some heating degradation. Another reason could be the heating promoting transformation of linoleic acid in 1-octen-3-ol, known as the alcohol of fungi, which is the principal aromatic compound in most fungi and might contribute to cooked mushrooms flavour (Maga, 1981).

Effects on the sugar composition

In what concerns sugar composition (**Table 3.4.3**) mushrooms showed some homogeneity. All of them presented mannitol and trehalose as main sugars, while other sugars were present either only in small amounts (*M. mastoidea* and *M. procera*) or were not detected (*L. deliciosus* and *S. imbricatus*).

Table 3.4.3. Sugar composition (g/100 g of dry weight) of the wild edible mushrooms (mean ± SD; n=3). In each row and for each species, different letters mean significant differences ($p<0.05$).

	Mannitol	Trehalose	Total sugars
L. deliciosus D	15.41 ± 1.90 a	0.88 ± 0.17 c	16.29 ± 1.75 a
L. deliciosus F	13.90 ± 0.41 a	3.50 ± 0.25 a	17.40 ± 0.66 a
L. deliciosus C	10.19 ± 0.13 b	2.24 ± 0.01 b	12.43 ± 0.13 b
M. mastoidea D	7.84 ± 0.14 a	4.11 ± 0.24 a	11.95 ± 0.20 a
M. mastoidea F	4.60 ± 0.97 b	5.11 ± 1.17 a	9.71 ± 2.11 ba
M. mastoidea C	7.54[a] ± 0.11 a	*nd*	7.54 ± 0.11 b
M. procera D	4.73 ± 0.26 b	2.92 ± 0.13 b	7.66 ± 0.40 b
M. procera F	6.51 ± 0.11 a	7.63 ± 0.58 a	14.13 ± 0.69 a
M. procera C	2.27[a] ± 0.45 c	1.19 ± 0.02 c	3.46 ± 0.44 c
S. imbricatus D	19.57 ± 1.62 a	6.03 ± 0.26 a	25.61 ± 1.87 a
S. imbricatus F	25.31 ± 1.48 a	4.99 ± 0.41 b	30.30 ± 1.77 a
S. imbricatus C	11.78 ± 1.72 b	3.44 ± 0.49 c	15.22 ± 2.21 b

[a] values corresponding to mannitol + glucose; D- dried; F-frozen; C- cooked.

nd- not detected.

The accumulation of these sugars in the fruit-bodies of other mushrooms such as *Hypsizygus marmoreus*, *Agaricus bisporus*, *Flammulina velutipes* and *Lentinus edodes* has been already reported (Harada et al., 2004). Mannitol was the most abundant sugar in all the studied species ranging from 2.27 g/100 g of dry weight for cooked *M. procera* and 25.31 g/100 g for frozen *S. imbricatus*. Other authors (Tan and Moore, 1994) also reported this sugar concentration up to 50% dry weight in *Agaricus bisporus* mushrooms. Sugar alcohols, particularly mannitol, function to provide support and expansion of the fruit body; this sugar alcohol has half the calories of sucrose, and is half as sweet, and being poorly absorbed by the body, it does not raise insulin levels as much as sugar and also does not promote tooth decay (Dikeman et al., 2005).

The values obtained for sugars concentrations were higher than those obtained for reducing sugars (**Table 3.4.1**) since trehalose, an abundant sugar in mushrooms fruiting

bodies, is an alpha-linked disaccharide and therefore non-reducing sugar. No significant differences between total sugars of dried and frozen samples were observed. Cooked samples presented significant lower sugar concentrations, expressed as dry weight, which is in agreement with the previously discussed for the carbohydrates results. It was also observed a significant decrease in trehalose concentration (glucose disaccharide), which probably was converted into glucose, due to the heating process. The chromatograms obtained for cooked *Macrolepiota* species presented broad peaks, probably including glucose and mannitol that have similar retention times. Nevertheless, the increase in glucose was not enough to balance the decrease in mannitol + glucose concentration caused by heat sugars degradation.

Effects on the antioxidant compounds

Table 3.4.4 shows EC_{50} values obtained in the antioxidant activity assays of wild edible mushrooms submitted to different conservation treatments and cooking, and also their concentrations in phenolic compounds. Dried samples revealed a higher content in total phenolics (ranging from 3.06 mg/g in *S. imbricatus* and 3.40 mg/g in *L. deliciosus*) and flavonoids (ranging from 0.99 mg/g in *M. procera* and 2.71 mg/g in *L. deliciosus*) compounds. It is important to note that flavonoids were determined by a colorimetric method that can also detect other compounds than flavonoids such as ortho-diphenols. The amounts found in the cooked samples significantly decreased when compared with the content found in the other samples. Some phenolic compounds are unstable and become non-antioxidative under heating; thus, heat used in the cooking procedure could destroy their structures and cause a decrease in their antioxidant activity (Leffer, 1993; Yen and Hung, 2000). Nevertheless, at low heating temperatures an increase in phenolics concentration may occur. This can be observed in the dried mushrooms that present higher phenols than the frozen samples (**Table 3.4.4**). Choi *et al.* (2006) described that heat treatment of Shiitake increased the overall content of 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 thus bound polyphenolic and flavonoid compounds may be released more easily relative to those of raw materials. Thermal processing is generally applied to extend shelf life of food products. It is also important to point out that other antioxidants besides phenolic compounds might be present in the extracts and contribute to their antioxidant properties.

Table 3.4.4. EC_{50} values obtained in the antioxidant activity assays and phenolic compounds contents in the wild edible mushrooms (mean ± SD; n=3). In each row and for each species, different letters mean significant differences ($p<0.05$).

	Antioxidant properties (EC_{50} values; mg/mL)				**Bioactive compounds (mg/g)**	
	DPPH scavenging activity	Reducing power	β-carotene bleaching inhibition	Lipid peroxidation inhibition	Total phenolics	Total flavonoids
L. deliciosus D	16.31 ± 0.24 c	4.98 ± 0.02 b	3.76 ± 0.24 b	26.40 ± 0.03 c	3.40 ± 0.18 a	2.71 ± 0.12 a
L. deliciosus F	20.54 ± 0.59 b	4.65 ± 0.02 b	3.74 ± 0.04 b	29.63 ± 0.08 b	2.95 ± 0.24 b	1.91 ± 0.07 b
L. deliciosus C	33.70 ± 1.69 a	17.66 ±0.08 a	17.86 ± 3.80 a	35.85 ± 0.03 a	1.23 ± 0.14 c	0.89 ± 0.06 c
M. mastoidea D	8.18 ± 0.07 c	4.35 ± 0.01 c	6.48 ± 1.10 b	24.20 ± 0.03 c	3.08 ± 0.17 a	2.10 ± 0.15 a
M. mastoidea F	8.49 ± 0.10 b	4.44 ± 0.04 b	8.92 ± 0.72 a	34.42 ± 0.05 b	2.69 ± 0.37 b	1.56 ± 0.11 b
M. mastoidea C	25.60 ± 0.13 a	4.79 ±0.03 a	8.10 ± 0.93 a	> 50 a	1.13 ± 0.09 c	1.08 ± 0.11 c
M. procera D	5.38 ± 0.50 c	4.18 ± 0.02 b	5.19 ± 0.16 b	> 50 a	3.17 ± 0.92 a	0.99 ± 0.05 a
M. procera F	6.95 ± 1.24 b	4.49 ±0.02 a	6.23 ± 1.23 ba	> 50 a	2.59 ± 0.17 a	0.90 ± 0.11 a
M. procera C	8.82 ± 0.36 a	3.96 ± 0.04 c	6.51 ± 1.08 a	> 50 a	2.45 ± 0.21 a	0.20 ± 0.11 b
S. imbricatus D	5.82 ± 0.06 c	4.41 ± 0.03 c	3.53 ± 0.20 c	38.17 ± 0.03 c	3.06 ± 0.10 a	1.52 ± 0.03 a
S. imbricatus F	10.98 ± 0.17 b	5.94 ± 0.05 b	4.45 ± 0.25 b	41.14 ± 0.03 b	2.22 ± 0.31 b	1.12 ± 0.03 b
S. imbricatus C	11.82 ± 0.20 a	8.74 ± 0.16 a	16.64 ± 1.08 a	44.42 ± 0.13 a	1.69 ± 0.38 c	1.02 ± 0.04 c

D- dried; F-frozen; C- cooked.

It is well-known that natural nutrients can be significantly lost during the thermal processing due to health instabilility. Therefore, heat processed foods may have a lower health promoting capacity than the corresponding fresh one. However, recent studies have shown that some thermally processed foods, especially fruits and vegetables, have higher biological activities due to their various chemical changes during heat treatment (Kim et al., 2000; Dewanto et al., 2002). In the present study the results showed that low heating temperatures (40 ºC) increased bioactive compounds content (dried samples) while higher heating temperatures destroyed those compounds (cooked samples).

The enhanced antioxidant activity in non-cooked samples could be explained by the increased amount of antioxidant compounds, particularly phenolic compounds (Ferreira et al., 2007). Also other authors described the loss of total antioxidant activity in blanched cruciferous vegetables. Yen and Hung (2000) suggested that alkali was an effective factor that caused a decrease in the antioxidative activity of the herb of *Mesona procumbens* Hemsl extracts, and that heating enhanced this effect. The authors attribute the decrease in antioxidant activity to the decrease in the content of the phenolic components. Other authors reported that heat treatment could also deactivate endogenous oxidative enzymes, explaining the increased antioxidant contents by preventing enzymatic oxidation causing loss of the antioxidant compound in the raw plant materials (Dewanto et al., 2002). This might explain the higher antioxidant activity (lower EC_{50} values) obtained for dried samples in comparison to frozen samples, in the present study. On the other hand, some antioxidant compounds can be present as a covalently bound form with insoluble polymers. Heat treatment might disrupt the cell wall and liberate bound forms from insoluble portion of mushroom, which, in turn, increases the pool of bioaccessible antioxidant compounds. Another reason for improved antioxidant activity could be due to formation of novel compounds having antioxidant activity during heat treatment or thermal processing (Choi et al., 2006).

Effects on the antioxidant properties

All the species proved to have antioxidant activity, namely radical scavenging activity and lipid peroxidation inhibition capacity. Nevertheless, different species revealed different properties according to each tested method, being dried samples more efficient (lower EC_{50} values), as it was already described. *M. mastoidea* cooked sample proved to

have lower antioxidant properties (higher EC_{50} values) which is compatible to its lower content of phenolic compounds. As an example, **Figure 3.4.1** shows the antioxidant activity of *S. imbricatus* after different treatment procedures examined as a function of their concentration. Several biochemical assays were used to screen the antioxidant properties: reducing power (measuring the conversion of a Fe^{3+}/ferricyanide complex to the ferrous form), scavenging activity on DPPH radicals (measuring the decrease in DPPH radical absorption after exposure to radical scavengers), inhibition of β-carotene bleaching (by neutralizing the linoleate-free radical and other free radicals formed in the system which attack the highly unsaturated β-carotene models), and inhibition of lipid peroxidation in brain tissue (measured by the colour intensity of MDA-TBA complex).

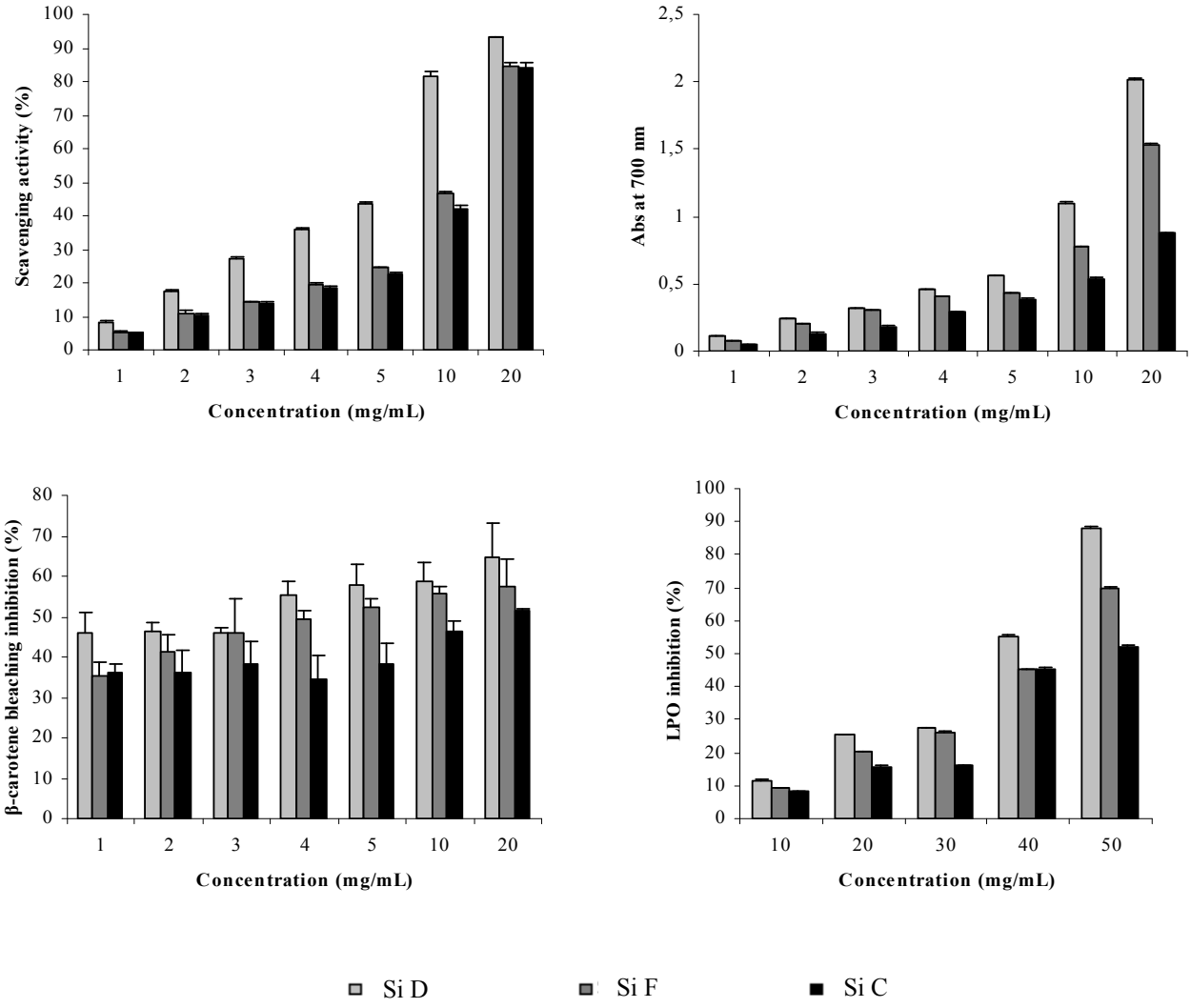


Figure 3.4.1. Antioxidant activity of *Sarcodon imbricatus* extracts: Scavenging activity on DPPH radicals (%), reducing power, hemolysis inhibition (%) and lipid peroxidation (LPO) inhibition (%). Each value is expressed as mean ± standard error (n=3).

The assays were performed in the whole extract, since it could be more beneficial than isolated constituents; a bioactive individual component can change its properties in the presence of other compounds present in the extracts (Borchers et al., 2004). According to Liu (2003) additive and synergistic effects of phytochemicals occur in fruits and vegetables and the benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals present in whole foods. This explains why no single antioxidant can replace the combination of natural phytochemicals to achieve the health benefits. Analysis of **Figure 3.4.1** revealed an antioxidant activity increased with the concentration increase, being obtained very good results at higher extracts concentrations. The antioxidant activity was better (lower EC_{50} values) in chemical assays than in the biochemical assay using animal cells (lipid peroxidation inhibition) (**Table 3.4.4**; **Figure 3.4.1**). The decrease in the antioxidant activity of cooked samples might be due to the destruction of phenolic hydroxyl groups due to high heat temperature.

In conclusion, both conservation treatment and cooking proved to have influence in mushrooms nutritional value and their antioxidant properties. Cooked samples proved to have lower nutrients concentrations and lower antioxidant activity either than dried or frozen samples. Nevertheless, for fatty acid and sugar individual profiles, only cooking procedure seems to be relevant, the cooked samples presenting higher MUFA, and lower PUFA and sugars contents.

3.4.2. Effect of *Lactarius piperatus* fruiting body maturity stage on antioxidant activity measured by several biochemical assays

3.4.2.1. Introduction

Oxidation is essential to many living organisms for the production of energy to fuel biological processes, proceeding in lipids with polyunsaturated fatty acids, and generating reactive oxygen species (ROS) such as hydroxyl radicals (Halliwell and Gutteridge, 1989). However, oxygen-centred free radicals and other ROS species, that are continuously produced in vivo as by products, result in cell death and tissue damage. The oxidative damage caused by free radicals may be related to aging and diseases, such as atherosclerosis, diabetes, cancer and cirrhosis (Halliwell and Gutteridge, 1984). Antioxidant compounds reduce the action of reactive oxygen species (ROS) in tissue damage.

Although humans and other organisms possess antioxidant defences (enzymes, such as superoxide dismutase and catalase, or compounds such as ascorbic acid, tocopherols and glutathione) and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to totally prevent the damage (Simic, 1988; Niki et al., 1994; Mau et al., 2002a).

Natural products with antioxidant activity are used to aid the endogenous protective system, increasing interest in the antioxidative role of nutraceutical products (Kanter, 1998). Concerning this, the antioxidants in human diets are of great interest as possible protective agents to help human body reduce oxidative damage. 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 (Ramarathnam et al., 1995). Mushrooms have also become attractive as a functional food and as sources for the development of drugs and nutraceuticals (Yanga et al., 2002). Mushrooms accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids. Phenolic compounds were found to have antioxidant activity in the inhibition of LDL oxidation (Teissedre and Landrault, 2000). Phenolics are one of the major groups of nonessential dietary components that have been associated with the inhibition of atherosclerosis and cancer (Williams and Iatropoulos, 1997). Flavonoids have proven to display a wide range of pharmacological and biochemical actions, such as antimicrobial, antithrombotic, antimutagenic and

anticarcinogenic activities (Cook and Samman, 1996; Kandaswami and Middleton, 1997; Sahu and Green, 1997).

Several studies (Yen and Hung, 2000; Mau et al., 2002a; Yang et al., 2002; Cheung et al., 2003; Mau et al., 2004; Cheung and Cheung, 2005; Lo and Cheung, 2005; Ferreira et al., 2006; Turkoglu et al., 2007) report a correlation between the mushrooms antioxidant activity and their phenolic content. However, none of the existent reports on mushrooms antioxidants composition and antioxidant activity indicated the stage of development of the fruiting bodies selected for the studies. Furthermore, no studies have been developed to evaluate the antioxidant activity at different stages of fruiting body maturity for potential use on the preparation of nutraceuticals.

In this study, we examined the evolution of antioxidant components and antioxidant activity of a *Basidiomycete* fungus, *L. piperatus*, in four stages of fruiting body maturity. The antioxidant properties were evaluated through reducing power, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, inhibition of oxidative hemolysis in erythrocytes induced by 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) and inhibition of lipid peroxidation by β-carotene-linoleate system. These assays have been extensively employed as models for the peroxidative damage in biomembranes. Antioxidant activity parameters have also been correlated to the phenolic, flavonoid, ascorbic acid, beta-carotene and lycopene contents.

3.4.2.2. Materials and methods

Standards and reagents

Standards BHA (2-*tert*-butyl-4-methoxyphenol), TBHQ (tert-butylhydroquinone), L-ascorbic acid, α-tocopherol, gallic acid and (+)-catechin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Folin and Ciocalteu's reagent is from Merck (Darmstadt, Germany). Methanol was obtained from Pronalab (Lisbon, Portugal). All other chemicals were obtained from Sigma. Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

Fruiting body selection

Wild fruiting bodies of *Lactarius piperatus* (L.) Pers. were obtained under live oak trees (*Quercus pyrenaica*), in Bragança (Northeast of Portugal), in spring 2006. Taxonomic identification was made according to several authors (Marchand, 1971; Moser, 1983; Bon, 1988; Courtecuisse and Duhem, 1995; Courtecuisse, 1999) and representative voucher specimens were deposited at the herbarium of *Escola Superior Agrária* of *Instituto Politécnico de Bragança*. The fruiting bodies were harvested and dried in different stages of maturity: immature (cap closed) and mature (cap opened). The mature stage was further characterized into immature spores and mature spores as seen on **Figure 3.4.2**.

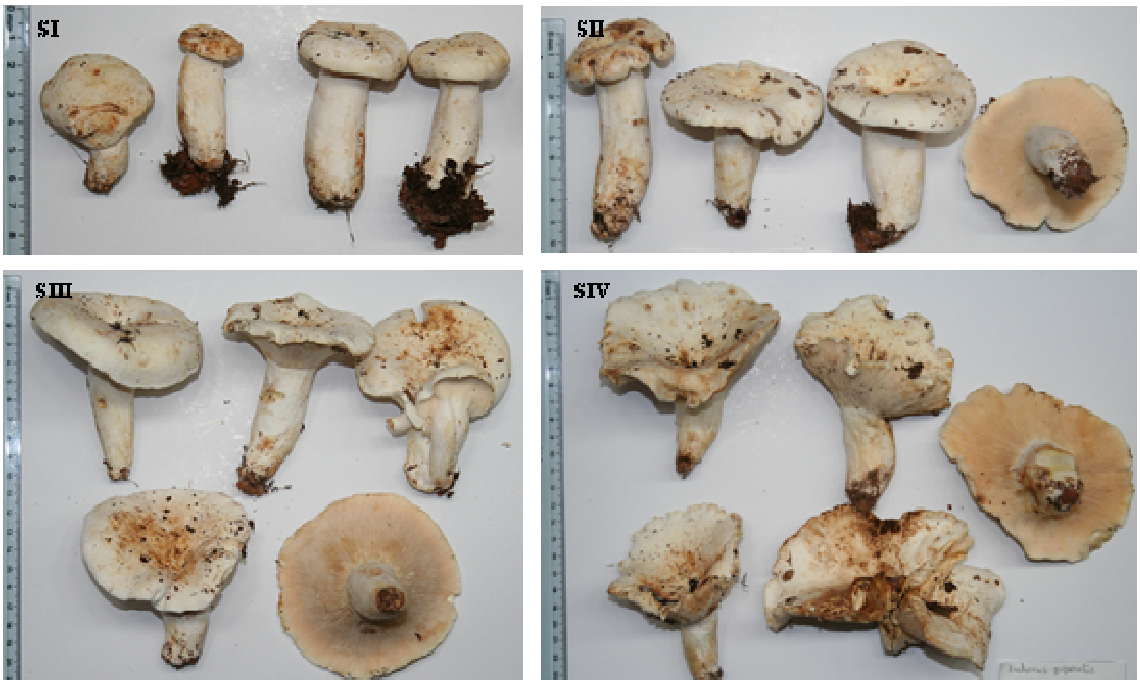


Figure 3.4.2. *Lactarius piperatus* fruiting bodies in different stages of maturity: (SI) immature (cap closed), (SII) mature (cap opened) with immature spores, (SIII) mature with mature spores, and (SIV) degraded.

Sample preparation

The fruiting bodies were air-dried in a liophylizator (Ly-8-FM-ULE, Snijders) and powdered before analysis. The dried samples (~5 g) were extracted by stirring with 100 mL of methanol at 25 ºC at 150 rpm for 24h and filtered through Whatman nº 4 paper. The residue was then extracted with two additional 100 mL portions of methanol, as described earlier. The combined methanolic extracts were evaporated at 40 ºC to

dryness and redissolved in methanol at a concentration of 50 mg/mL, and stored at 4 ºC for further use.

Determination of total antioxidant components

Phenolics, flavonoids, ascorbic acid, β-carotene and lycopene contents were determined as described in **3.1.4.2** (*Total bioactive compounds*).

Antioxidant activity assays

DPPH radical-scavenging activity, reducing power, inhibition of erythrocyte hemolysis mediated by peroxyl free radicals and inhibition of lipid peroxidation using the β-carotene linoleate model system were evaluated as described in **3.2.1.2**.

Statistical analysis

All the determinations were carried out in triplicate and the results are expressed as mean values and standard deviations (SD). Differences among means were done by analysis of variance (ANOVA), using SAS v. 9.1.3, and averages were compared using Tukey test ($p < 0.05$). A regression analysis, using Excel for Windows Software, was established between phenolic and flavonoid contents of different mushroom samples and EC_{50} values obtained by different antioxidant assays.

3.4.2.3. Results and discussion

Table 3.4.5 shows total phenolics, flavonoid, ascorbic acid and carotenoids concentrations obtained in *L. piperatus* in different stages of fruiting body maturity. Whereas phenolics were the major antioxidant components found in the extracts, ascorbic acid was found in small amounts (0.03–0.16 mg/g), and β-carotene and lycopene were only found in vestigial amounts (<34μg/g), which is in agreement with other previous reports concerning ascorbic acid and β-carotene quantification in different mushrooms (Mau et al., 2002a), as well as the results obtained by us in previous studies with other mushroom species (see **3.2.1.**).

Table 3.4.5. Total phenolics, flavonoids, ascorbic acid, β-carotene and lycopene contents (mean ± SD) of *Lactarius piperatus* in different stages of growth. In each row different letters mean significant differences ($p<0.05$).

	Stage I	Stage II	Stage III	Stage IV
Total phenolics (mg/g)	5.52± 0.14 b	5.76±0.09 a	3.09±0.12 c	2.03±0.11 d
Flavonoids (mg/g)	1.26±0.09 b	1.58±0.02 a	0.35±0.03 c	0.19±0.01 d
Ascorbic acid (mg/g)	0.15±0.01 a	0.16±0.01 a	0.13±0.01 b	0.03±0.01 c
β-carotene (μg/g)	26.08±0.05 b	33.78±0.05 a	17.22±0.00 c	15.11±0.02 d
Lycopene (μg/g)	8.14±0.03 b	13.04±0.02 a	5.80±0.01 c	5.41±0.01 d

Curiously, the stages where the fruiting bodies presented immature spores (stage I and II) revealed a higher content in phenolic and flavonoid compounds. The amount found in stages III (with mature spores) and IV (degraded) significantly ($p<0.05$) decreased when compared with the content found in the first stages. Probably, the aging process elicits the formation of reactive oxygen species, which are neutralised by the phenolic compounds, resulting in the lowering of their contents and antioxidant capacities in the most advanced stages. The highest content of antioxidant compounds in the first stages might account for the better results found in their antioxidant activity (Velioglu et al., 1998; Ferreira, 2007). Furthermore, Cheung et al. (2003) found a direct correlation between mushrooms antioxidant activity and total phenolic content, although other substances such as tocopherols and β-carotene also account for the antioxidant action. The bioactivity of phenolics may be related to their ability to chelate metals, inhibit lipoxygenase and scavenge free radicals (Decker, 1997). Also, in food systems, flavonoids can act as free radical scavengers and terminate the radical chain reactions that occur during the oxidation of triglycerides, presenting antioxidative efficiency in oils, fats and emulsions (Madhavi et al., 1996; Roedig-Penman and Gordon, 1998).

Figure 3.4.3 shows the antioxidant activity of *L. piperatus* in different stages of fruiting body maturity, measured by different biochemical assays.

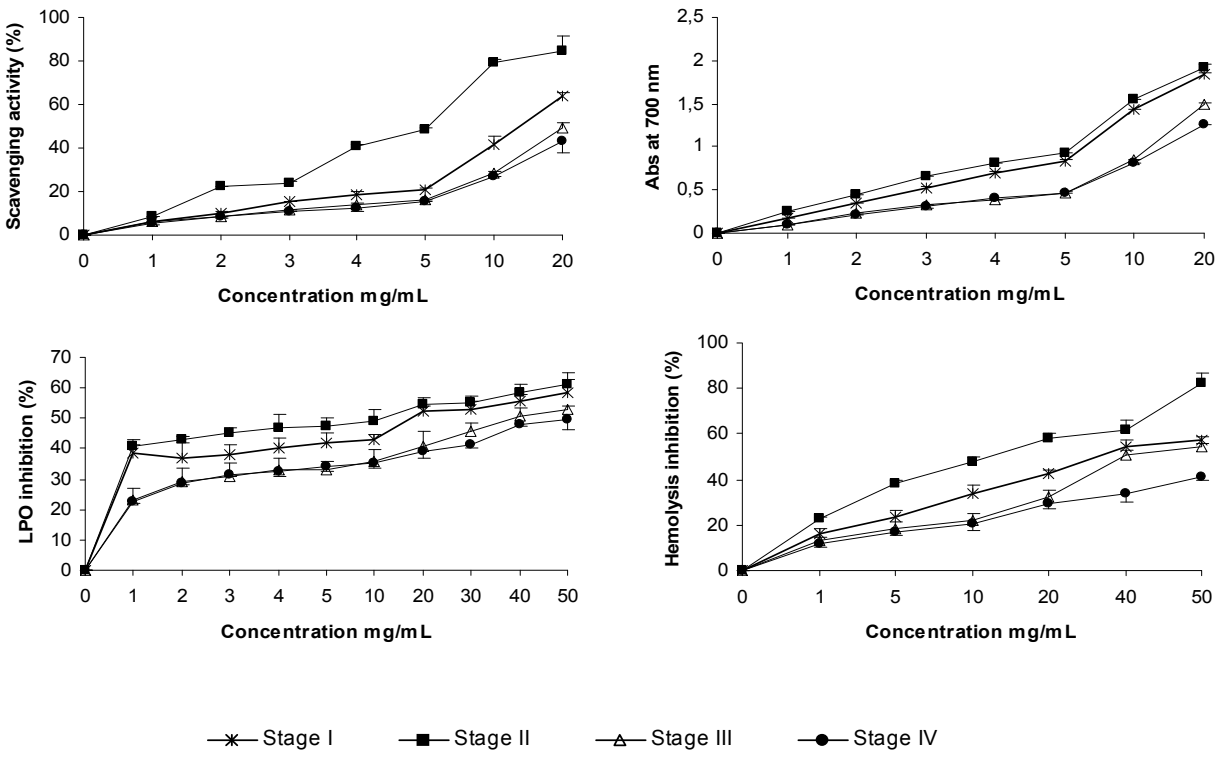


Figure 3.4.3. Antioxidant activity of *Lactarius piperatus* in different stages of growth assessed by different assays: scavenging activity on DPPH radicals (%), reducing power, hemolysis inhibition (%) and lipid peroxidation (LPO) inhibition (%). Each value is expressed as mean ± standard error (n=3).

The radical scavenging activity (RSA) values were expressed as the ratio percentage of sample absorbance decrease and the absorbance of DPPH solution in the absence of extract at 517 nm. From the analysis of **Figure 3.4.3.**, we can conclude that the scavenging effects of fruiting body extracts on DPPH radicals increased with the concentration increase and were very good for stage II (84.9% at 20 mg/mL), but lower than the scavenging effects of the standards BHA (96% at 3.6 mg/mL) and α-tocopherol (95% at 8.6 mg/mL). The RSA values at 20 mg/mL were moderate for the other stages (stage I - 64.2%, stage III - 49.4%, stage IV - 43.4%) and, particularly stage III and IV revealed a very similar scavenging activity.

The reducing power also increased with concentration, and the values obtained for all the extracts were excellent (**Figure 3.4.3.**); at 20 mg/mL were higher than 1.3 and in the order of stage II > stage I > stage III > stage IV. Reducing power of BHA at 3.6 mg/mL and α-tocopherol at 8.6 mg/mL was only 0.12 and 0.13, respectively. The extracts obtained in stages III and IV showed similar reducing power values. It was reported that the reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by

donating a hydrogen atom (Shimada at al., 1992). Accordingly, in stage II, it is supposed that higher amounts of reductones were produced, which could react with free radicals to stabilize and block radical chain reactions.

In this study, the protective effect of the fruiting body extracts on hemolysis by peroxyl radical scavenging activity was also investigated. AAPH is a peroxyl radical initiator that generates free radicals by its thermal decomposition and attack the erythrocytes to induce the chain oxidation of lipid and protein, disturbing the membrane organization and eventually leading to hemolysis. The extracts inhibited hemolysis, as a result of protection against the oxidative damage of cell membranes of erythrocytes induced by AAPH, in a concentration-dependent manner (**Figure 3.4.3**). Once more, in stage II there was a higher protective effect against erythrocytes hemolysis (82.4% at 50 mg/mL) than in other stages (stage I - 57.5%, stage III - 54.2%, stage IV - 41.0%). However, the inhibition percentage of the standard L-ascorbic acid on hemolysis of red blood cell was much higher (94.6% at 1mg/mL) than those of mushroom extracts.

The lipid peroxidation inhibition (LPO), measured by the bleaching of β-carotene, is presented in **Figure 3.4.3**. The linoleic acid free radical attacks the highly unsaturated β-carotene models. The presence of different antioxidants can hinder the extent of β-carotene-bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system (Jayaprakasha et al., 2001). Accordingly, the absorbance decreased rapidly in samples without antioxidant, whereas in the presence of an antioxidant, they retained their colour, and thus absorbance, for a longer time. LPO inhibition in the presence of *L. piperatus* in different stages of fruiting body maturity increased with their increasing concentration, and the values at 50 mg/mL for each one of the stages were 58.3, 61.2, 52.9 and 49.72 %. It is probable that the antioxidative components in the mushroom extracts can reduce the extent of β-carotene destruction by neutralizing the linoleate free radical and other free radicals formed in the system. Again, stage II was the most effective for antioxidant activity. The antioxidant activity of TBHQ standard reached 82.2% at 2 mg/mL and was more efficient than the samples. Nevertheless, this and other synthetic antioxidants applied in fat and oily foods to prevent oxidative deterioration were found to be carcinogenic in experimental animals (Loliger, 1991).

In **Table 3.4.6** EC_{50} values obtained in the antioxidant activity assays of *L. piperatus* in different stages of fruiting body maturity are shown. Overall, the mushroom in stage II

revealed better antioxidant properties (significantly lower EC_{50} values; $p<0.05$) than in other stages, which is in agreement with the higher content of antioxidants found in the first case. The EC_{50} values obtained for reducing power and scavenging effects on DPPH radicals were better than for LPO inhibition and for hemolysis inhibition mediated by peroxyl free radicals.

Table 3.4.6. EC_{50} values obtained in the antioxidant activity assays of *Lactarius piperatus* in different stages of growth. In each row different letters mean significant differences ($p<0.05$).

EC_{50} values (mg/L)	Stage I	Stage II	Stage III	Stage IV
Scavenging effect	13.92±0.73 c	5.19±0.03 d	20.24±0.78 b	23.44±3.33 a
Reducing power	2.83±0.01 c	2.29±0.02 d	5.40±0.05 b	5.50±0.03 a
Hemolysis inhibition	32.92±0.72 c	12.14±0.03 d	38.91±0.18 b	>50.0 a
LPO inhibition	15.26±4.15 c	12.08±3.15 c	34.25±5.46 b	45.06±2.21 a

Significantly negative linear correlations were established between the contents of phenolic compounds and flavonoids, and EC_{50} values of DPPH scavenging activity (determination coefficient 0.806 for phenolics and 0.868 for flavonoids; $p < 0.001$), reducing power (determination coefficient 0.946 for phenolics and 0.986 for flavonoids; $p < 0.001$), hemolysis inhibition (determination coefficient 0.792 for phenolics and 0.798 for flavonoids; $p < 0.001$) and LPO inhibition (determination coefficient 0.940 for phenolics and 0.914 for flavonoids; $p<0.001$) (**Figure 3.4.4**). Nevertheless it is important to note that flavonoids were determined by a colorimetric method that can also detect other compounds than flavonoids such as ortho-diphenols. These negative linear correlations show that the sample with highest phenolic contents shows higher antioxidant activity and lower EC_{50} values (stage II, mature with immature spores), while the sample with lowest phenolic contents presents lower antioxidant activity and higher EC_{50} values (stage IV, degraded). The correlations also support that the mechanism of action of the extracts for the antioxidant activity may be identical, being related with the content in phenolics and flavonoids, and their free-radical scavenging activity.

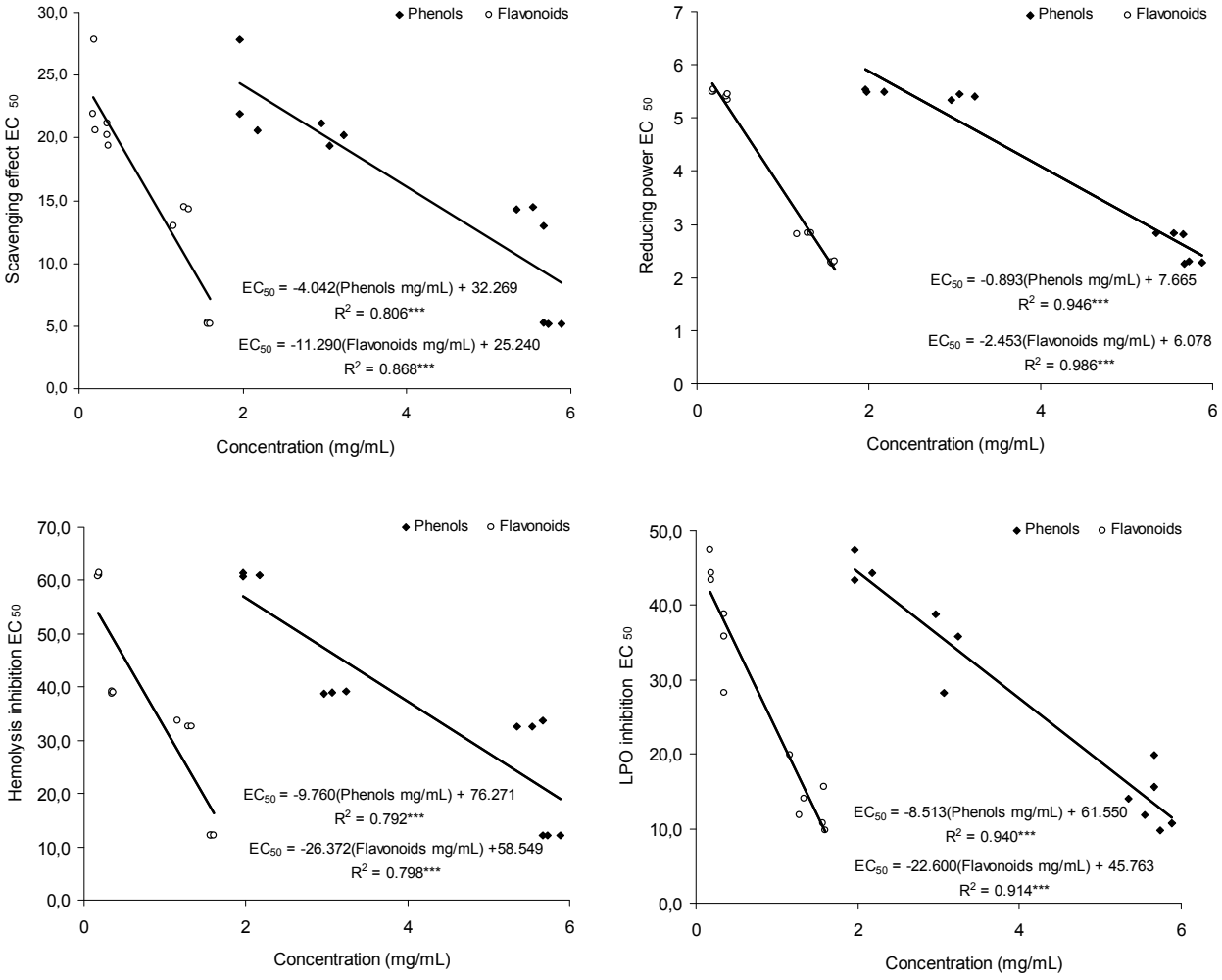


Figure 3.4.4. Correlations established between total phenol and flavonoid contents, and scavenging effect on DPPH radicals, reducing power, hemolysis inhibition and LPO inhibition.

Over two-thirds of cancer-related death could be prevented through lifestyle modification, particularly through dietary means and, mushrooms consumption, could contribute to minimize cancer risks through antioxidants input. Nevertheless, it is important to know the best maturity stage to collect wild mushrooms concerning antioxidants production and, to our best knowledge, the present study was the first report to demonstrate that the antioxidative components production by wild mushrooms and their antioxidant properties depends on the stage of fruiting body maturity.

3.4.3. Effect of fruiting body maturity stage on chemical composition and antimicrobial activity of Lactarius sp. mushrooms

3.4.3.1. Introduction

In recent years, multiple drug resistance in human pathogenic microorganisms has developed, due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. This situation has forced scientists to search for new antimicrobial substances from various plants as sources of novel antimicrobial chemotherapeutic agents (Karaman et al., 2003; Turkoglu et al., 2007). Mushrooms are rich sources of natural antibiotics, and therefore mushrooms extracts have been investigated for their antimicrobial activity. It was demonstrated the capacity of the Chinese Shiitake mushroom (*Lentinus edodes*) to increase the host resistance to bacterial and viral infections (Jong and Birmingham, 1993) and several compounds extracted from this mushroom revealed antifungal and antibacterial activity (Yasumoto et al., 1971; Takasawa et al., 1982; Hirasawa et al., 1999; Hatvani et al., 2001; Kritzberger et al., 2007). *Laetiporus sulphureus* antimicrobial activity was also reported and correlated to the phenolics and flavonoids contents (Turkoglu et al., 2007). Similar results were obtained by us on the antimicrobial properties of phenolic extracts of other Portuguese wild edible mushroom species (*Lactarius deliciosus*, *Sarcodon imbricatu*s and *Tricholoma portentosum*) (see chapter **3.2.3**). Additionally, many of the externalized secondary metabolites (extracellular secretions by the mycelium) combat bacteria (Benedict and Brady, 1972; Kupra et al., 1979), viruses (Eo et al., 199; Brandt and Piraino, 2000), and protozoa (Lovy et al., 1999; Isawka et al., 2001). Compounds such as phenolics (phenolic acids and flavonoids), carotenoids, tocopherols and ascorbic acid are important protective agents for human health (Block et al.,1992).

Besides their pharmacological characteristics (Bobek et al., 1991; Bobek et al., 1999), wild mushrooms are becoming more and more important in our diet for their nutritional properties, including relatively high protein and mineral contents, and low fat/energy levels (Aletor, 1995; Longvah and Deosthale, 1998). Dikeman et al. (2005) reported the effects of stage of maturity and cooking on the chemical composition of mushroom species commonly cultivated and consumed in the United States. Nevertheless, there are no detailed studies on the influence in fatty acid and sugars profiles, particularly on mushrooms from Northeast Portugal, one of the European regions with higher wild

edible mushrooms diversity. Also, none of the existent reports on mushrooms antimicrobial activity and bioactive compounds indicated the stage of development of the fruiting bodies selected for the studies. The nutraceutical quality of a mushroom is dependent on the chemical composition of the fruiting body (Camelini et al., 2005) and, therefore, it is important to develop studies to evaluate bioactive properties and chemical composition at different stages of fruiting body maturity either for potential use on the preparation of nutraceutical, or to achieve the ideal stage for consumption concerning nutritional value.

In this study, we examined the evolution of chemical composition and antimicrobial activity of Portuguese *Lactarius* species (*L. deliciosus* and *L. piperatus*) in three stages of fruiting body maturity. Chemical composition evaluation included moisture, fat, protein, ash, carbohydrates, and individual fatty acid and sugar profiles, as well as bioactive compounds determination such as phenolics, flavonoids and carotenoids. Antimicrobial activity was screened against Gram positive and Gram negative bacteria and fungi, and correlated to the levels of bioactive compounds present in the extracts. The entire mushroom extracts were used in these assays to achieve health benefits due to the additive and/or synergistic effects of all the bioactive compounds present.

3.4.3.2. Materials and methods

Samples/Fruiting bodies selection

Wild fruiting bodies of *Lactarius deliciosus* (L.) Gray and *Lactarius piperatus* (L.) Pers. were obtained, respectively, under live pine (*Pinus* sp.) in Autumn 2005 and oak (*Quercus pyrenaica*) trees in Spring 2006, in Bragança (Northeast of Portugal). Taxonomic identification was made according to several authors (Moser, 1983; Courtecuisse and Duhem, 1995) and representative voucher specimens were deposited at the herbarium of *Escola Superior Agrária* of *Instituto Politécnico de Bragança*. The fruiting bodies were harvested and dried in different stages of maturity: immature and mature (with different cap diameters), and the mature stage was further characterized into immature spores and mature spores (**Figure 3.4.5)**.

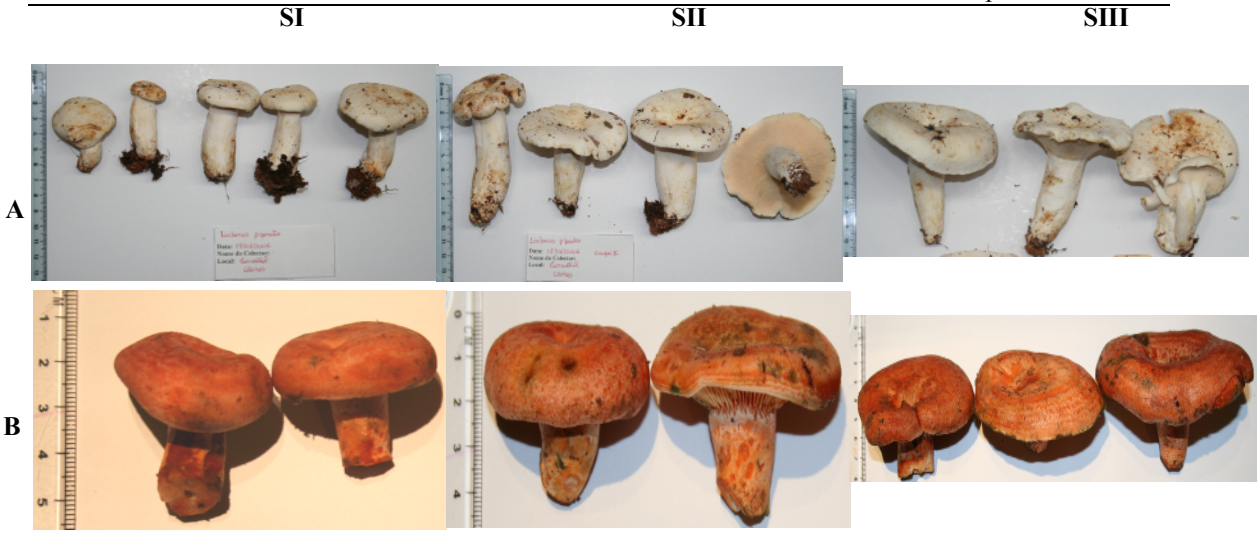


Figure 3.4.5. *Lactarius piperatus* (**A**) and *Lactarius deliciosus* (**B**) fruiting bodies in different stages of maturity: (SI) immature (cap diameter less than 4.5 cm), (SII) mature (cap diameter between 4.5 and 7 cm) with immature spores, and (SIII) mature (cap diameter higher than 7 cm) with mature spores.

Standards and reagents

All reagents were of analytical grade purity: methanol and diethyl ether were supplied by Lab-Scan (Lisbon, Portugal); toluene from Riedel-de-Haën (Hanover, Germany); sulphuric acid from Fluka (St. Gallen, Switzerland). The fatty acids methyl ester (FAME) reference standard mixture 37 (fatty acids C4 to C24; (standard 47885-U) was from Supelco (Bellefonte, PA, USA) and from Sigma Chemical Co. (St. Louis, MO, USA), as also other individual fatty acid isomers, gallic acid and (+)-catechin. The standards used in the antimicrobial activity assays, ampicillin and cycloheximide, as while as Folin and Ciocalteu's reagents were purchased from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma. Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

Chemical Composition

Samples of mushrooms were analysed for chemical composition (moisture, protein, fat, carbohydrates and ash) using as previously described in **3.1.1.2**. (*Chemical composition*). Reducing sugars were determined by DNS (dinitrosalicylic acid) method.

Fatty Acid Composition. Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GLC-FID)/capillary column as described in **3.1.4.2**. (*Fatty acid composition*).

Sugar Composition. Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) as described in **3.1.1.2**. (*Sugar composition*).

Antimicrobial activity

Sample preparation. The samples (~3 g) were extracted by stirring with 100 mL of methanol at 25 ºC at 150 rpm for 24h and filtered through Whatman nº 4 paper. The residue was then extracted with two additional 100 mL portions of methanol, as described earlier. The combined methanolic extracts were evaporated at 40 ºC to dryness and redissolved in DMSO at a concentration of 50 mg/mL, and stored at 4 ºC for further use.

Microorganisms and culture conditions. Microorganisms labeled CECT were obtained from the Spanish type culture collection (CECT) of Valencia University, while microorganisms labeled ESA were clinically isolated strains from different biological fluids, identified by Microbiology Laboratory of *Escola Superior Agrária de Bragança*. Gram + (*Bacillus cereus* CECT 148, *B. subtilis* CECT 498, *Staphylococus aureus* ESA 7 isolated from pus) and Gram – (*Escherichia coli* CECT 101, *Pseudomonas aeruginosa* CECT 108, *Klebsiella pneumoniae* ESA 8 isolated from urine) bacteria, and fungi (*Candida albicans* CECT 1394, *Cryptococcus neoformans* ESA 3 isolated from vaginal fluid) were used to screen samples antimicrobial activity. Microorganisms were cultured aerobically at 37 ºC (Scientific 222 oven model) in nutrient agar medium for bacteria, and at 30 ºC in sabouraud dextrose agar medium for fungi.

Test assays for antimicrobial activity. A screening of antibacterial activities against the Gram - and Gram + bacteria and fungi was performed, following the procedure described in **3.1.4.2** (*Test assays for antimicrobial activity*).

Determination of bioactive components.

Phenolics, flavonoids, ascorbic acid, β-carotene and lycopene were determined as described in **3.1.4.2**. (*Total bioactive compounds*).

Statistical analysis

For each one of the fruiting body maturity stages three samples were analysed and also all the assays were carried out in triplicate. The results are expressed as mean values and standard error (SE) or standard deviation (SD). The effects of the fruiting body maturity stage on the chemical composition and antimicrobial activity were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SAS v. 9.1.3 program.

3.4.3.3 Results and discussion

Effects on the chemical composition

The results of the chemical composition and estimated energetic value obtained for the studied wild mushroom species are shown in **Table 3.4.7**. Concerning moisture, fat and ash contents, no significant differences were obtained between the samples at different stages of fruiting body maturity. The most abundant macronutrients (carbohydrates and proteins) presented opposite evolutions along the maturity of fruiting body. Carbohydrates significantly decreased in both species (7.32 to 2.96 g/100g for *L. deliciosus* and 9.35 to 4.29 g/100g for *L. piperatus*) while proteins increased (1.29 to 4.41 g/100g for *L. deliciosus* and 0.82 to 4.12 g/100g for *L. piperatus*).

The decrease in carbohydrates content may be explained by their energetic role, being catabolised for energy production along the mushroom growth. Otherwise, the increase in proteins (mainly structural compounds) content may be due to the protein synthesis inherent to mushrooms maturity (Lehninger, 2005).

On the basis of the proximate analysis, it can be calculated that 100g of fresh *L. piperatus* assures higher energy values than *L. deliciosus*, in the same stage of maturity. the highest values are guaranteed by immature fruiting bodies (**Table 3.4.7**), despite the similarity in the results obtained for the other maturity stages. Concerning dry matter, there are no significant differences between immature and mature fruiting bodies. This is in agreement with other authors (Dikeman et al., 2005) which reported that the stage of maturity did not affect mushrooms proximate constituents in a consistent manner.

Table 3.4.7. Moisture, proximate chemical composition (g/100 g of fresh weight) and energetic value (Kcal/100 g of fresh weight) of the mushrooms in different stages of maturity (mean ± SD; n=3). In each row and for each species, different letters mean significant differences ($p<0.05$).

Samples	Moisture	Total fat	Crude protein	Ash	Carbohydrates	Reducing sugars	Energy
L. deliciosus SI	90.91 ± 0.27 a	0.47 ± 0.21 a	1.29 ± 0.02 c	1.30 ± 0.03 b	7.32 ± 0.99 a	0.05 ± 0.00 b	38.68 ± 2.60 a
L. deliciosus SII	90.55 ± 0.58 ba	0.37 ± 0.10 a	2.07 ± 0.08 b	1.44 ± 0.02 a	5.21 ± 0.18 b	0.18 ± 0.02 a	32.41 ± 1.36 b
L. deliciosus SIII	89.61 ± 0.31 b	0.55 ± 0.10 a	4.41 ± 0.07 a	1.54 ± 0.07 a	2.96 ± 0.50 c	0.04 ± 0.00 b	34.39 ± 2.29 ba
L. piperatus SI	88.04 ± 1.05 a	0.97 ± 0.16 a	0.82 ± 0.02 c	0.83 ± 0.05 a	9.35 ± 1.10 a	0.05 ± 0.00 b	49.41 ± 3.82 a
L. piperatus SII	89.28 ± 0.33 a	0.69 ± 0.32 a	1.58 ± 0.03 b	0.94 ± 0.15 a	7.51 ± 0.18 a	0.22 ± 0.02 a	42.53 ± 3.50 ba
L. piperatus SIII	89.90 ± 1.17 a	0.70 ± 0.20 a	4.12 ± 0.14 a	0.99 ± 0.20 a	4.29 ± 1.36 b	0.06 ± 0.00 b	39.96 ± 4.29 b

The results for fatty acid composition, total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) of the studied mushrooms are shown in **Table 3.4.8**. For both species, the major fatty acid found was stearic acid (C18:0), followed by linoleic acid (C18:2) and oleic acid (C18:1). Besides those three main fatty acids, nineteen more were identified and quantified. Saturated fatty acids (SFA) predominate over the unsaturated fatty acids (UFA) in all the stages of fruiting body maturity. SFA content decreased with the increase of fruiting body maturity due to the decrease in stearic acid percentage. Otherwise, the increase in linoleic and oleic acids contribute to the overall increase in PUFA and MUFA contents, respectively.

In what concerns sugar composition (**Table 3.4.9**) mushrooms with fruiting bodies in different maturity stages showed some homogeneity. All of them presented mannitol and trehalose as main sugars. Mannitol was the most abundant sugar for *L. deliciosus* mushrooms being trehalose predominant in *L. piperatus* samples. A slightly increase in mannitol content was observed for mature stages. Sugar alcohols, particularly mannitol, function to provide support and expansion of the fruit body, explaining the increase in sugar alcohol concentration with maturity of the fruiting body as observed in the current study.

Table 3.4.8. Fatty acid composition (percent) of the mushrooms in different stages of maturity (mean ± SD; n=3). In each row and for each species, different letters mean significant differences ($p<0.05$).

	L. deliciosus SI	*L. deliciosus* SII	*L. deliciosus* SIII	*L. piperatus* SI	*L. piperatus* SII	*L. piperatus* SIII
C10:0	4.67 ± 0.76	5.46 ± 0.58	3.84 ± 0.81	0.03 ± 0.00	0.06 ± 0.02	0.09 ± 0.02
C11:0	*nd*	*nd*	*nd*	0.01 ± 0.00	0.02 ± 0.01	0.03 ± 0.01
C12:0	0.01 ± 0.00	0.41 ± 0.01	0.50 ± 0.09	0.01 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
C14:0	0.32 ± 0.07	0.18 ± 0.03	0.23 ± 0.03	0.11 ± 0.01	0.17 ± 0.02	0.24 ± 0.03
C14:1	0.17 ± 0.03	0.11 ± 0.01	0.05 ± 0.01	*nd*	*nd*	*nd*
C15:0	0.32 ± 0.06	0.32 ± 0.02	0.32 ± 0.02	0.28 ± 0.03	0.37 ± 0.03	0.51 ± 0.04
C16:0	4.19 ± 0.36	5.40 ± 0.13	7.02 ± 0.34	1.10 ± 0.13	2.16 ± 0.10	5.15 ± 0.64
C16:1	0.11 ± 0.01	0.18 ± 0.01	0.30 ± 0.02	0.03 ± 0.00	0.06 ± 0.00	0.12 ± 0.02
C17:0	0.17 ±0.01	0.15 ± 0.00	0.11 ± 0.00	0.16 ± 0.01	0.14 ± 0.00	0.12 ± 0.01
C17:1c	*nd*	*nd*	*nd*	0.01 ± 0.00	0.08 ± 0.02	0.18 ±0.07
C18:0	61.71 ±2.43	51.29 ± 0.30	37.82 ± 1.08	81.46 ± 1.45	74.97 ± 0.51	54.53 ± 2.84
C18:1n9c	5.50 ± 0.67	12.51 ± 0.40	22.93 ± 0.44	6.58 ± 0.72	11.35 ± 0.40	26.99 ± 2.33
C18:2n6c	21.43 ± 0.84	22.70 ± 0.34	25.37 ± 0.10	6.66 ± 0.59	6.86 ± 0.04	8.57 ± 0.17
C18:3n6	0.26 ± 0.10	0.18 ± 0.03	0.15 ± 0.04	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
C18:3n3	0.34 ± 0.04	0.37 ± 0.01	0.48 ± 0.02	0.04 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C20:0	0.17 ± 0.01	0,18 ± 0.01	0.15 ± 0.01	2.40 ± 0.03	2.18 ± 0.03	1.56 ± 0.14
C20:1c	0.09 ± 0.00	0,03 ± 0.00	0.04 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.04 ± 0.00
C20:2c	0.04 ± 0.01	0,04 ± 0.00	0.04 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C20:3n3+C21:0	0.02 ± 0.00	0,02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
C22:0	0.15 ± 0.06	0,16 ± 0.04	0.21 ± 0.02	0.39 ± 0.01	0.53 ± 0.03	0,52 ± 0.02
C23:0	0.07 ± 0.02	0,05 ± 0.01	0.06 ± 0.01	0.17 ± 0.02	0.17 ± 0.01	0.48 ± 0.10
C24:0	0.26 ± 0.06	0,27 ± 0.10	0.35 ± 0.02	0.53 ± 0.02	0.76 ± 0.02	0.76 ± 0.02
total SFA	72.04 ± 3.83 a	63.87 ± 1.22 b	50.60 ± 2.42 c	86.62 ± 1.72 a	81.53 ± 0.77 b	64.00 ± 3.86 c
total MUFA	5.87 ± 0.72 c	12.83 ± 0.42 b	23.32 ± 0.47 a	6.63 ± 0.73 c	11.51 ± 0.43 b	27.33 ± 2.42 a
total PUFA	22.09 ± 0.99 c	23.30 ± 0.38 b	26.06 ± 0.17 a	6.74 ± 0.59 b	6.94 ± 0.05 b	8.64 ± 0.18 a

Table 3.4.9. Sugar composition (g/100 g of fresh weight) of the mushrooms in different stages of maturity (mean ± SD; n=3). In each row and for each species, different letters mean significant differences ($p<0.05$).

	Mannitol	Trehalose	Total sugars
L. deliciosus SI	1.24 ± 0.01 b	0.35 ± 0.00 a	1.59 ± 0.01 a
L. deliciosus SII	1.34 ± 0.10 ba	0.38 ± 0.03 a	1.72 ± 0.13 a
L. deliciosus SIII	1.50 ± 0.07 a	0.24 ± 0.01 b	1.74 ± 0.08 a
L. piperatus SI	0.36 ± 0.07 a	1.16 ± 0.10 a	1.51 ± 0.12 a
L. piperatus SII	0.41 ± 0.02 a	0.83 ± 0.20 a	1.24 ± 0.18 a
L. piperatus SIII	0.43 ± 0.04 a	1.20 ± 0.17 a	1.63 ± 0.21 a

No significant differences between total sugars of immature or mature stages were observed, being only produced a slight increase in mature fruiting bodies. In fact, other authors (*25*) observed for different species (*Grifola frondosa* and *Lentinus edodes*) a total free monosaccharides increase in mature mushrooms, as compared with immature mushrooms, although the same results were not found for *Agaricus bisporus*.

Effects on the antimicrobial activity

Table 3.4.10 shows total phenolics, flavonoids, ascorbic acid and carotenoids concentrations obtained in *Lactarius* species in different stages of fruiting body maturity. Whereas total phenolics were the major bioactive components found in the extracts, ascorbic acid was found in small amounts (0.08–0.16 mg/g), and β-carotene and lycopene were only found in vestigial amounts (<49 μg/g).

Table 3.4.10. Bioactive compounds contents of the mushrooms in different stages of maturity (mean ± SD; n=3). In each row and for each species, different letters mean significant differences ($p<0.05$).

Sample	Total phenolics (mg/g)	Flavonoids (mg/g)	Ascorbic acid (mg/g)	β-carotene (μg/g)	Lycopene (μg/g)
L. deliciosus SI	5.84 ± 0.34 a	2.58 ± 0.09 a	0.11 ± 0.01 a	48.35 ± 0.40 a	32.63 ± 0.29 a
L. deliciosus SII	4.55 ± 0.24 b	2.20 ± 0.02 b	0.10 ± 0.00 b	32.37 ± 0.31 b	22.18 ± 0.22 b
L. deliciosus SIII	3.02 ± 0.11 c	1.84 ± 0.12 c	0.08 ± 0.00 c	26.20 ± 0.24 c	20.23 ± 0.17 c
L. piperatus SI[a]	5.52 ± 0.14 b	1.26 ± 0.09 b	0.15 ± 0.01 a	26.08 ± 0.05 b	8.14 ± 0.03 b
L. piperatus SII[a]	5.76 ± 0.09 a	1.58 ± 0.02 a	0.16 ± 0.01 a	33.78 ± 0.05 a	13.04 ± 0.02 a
L. piperatus SIII[a]	3.09 ± 0.12 c	0.35 ± 0.03 c	0.13 ± 0.01 b	17.22 ± 0.00 c	5.80 ± 0.01 c

[a] see chapter **3.4.2**.

The mature stage where the fruiting bodies presented mature spores (stage III) revealed a lower content in bioactive compounds. For both species, the amount found in stage III significantly (p<0.05) decreased when compared with the content found in the first stages. Probably, those compounds are involved in defence mechanisms inherent to the aging process (presence of mature spores), resulting in the lowering of their contents in the most advanced stage. Otherwise, stages I and II did not reveal the same profile for the different *Lactarius* species. A higher content in bioactive compounds were found in stage I (immature fruiting bodies) for *L. deliciosus*, while for *L. piperatus*, stage II (mature with immature spores) presented the highest content.

Table 3.4.11 shows the antimicrobial screening of *L. deliciosus* and *L. piperatus* in different stages of fruiting body maturity against *B. cereus*, *B. subtilis*, *S. aureus* (Gram +), *E. coli*, *P. aeruginosa*, *K. pneumoniae* (Gram -) bacteria, and *C. albicans* and *C. neoformans* (fungi). The MICs for bacteria and fungi were determined as an evaluation of the antimicrobial activity of the tested mushrooms. The relative size of the halos of the inhibition zones corresponding to the MICs are also presented. All the stages of maturity revealed antimicrobial activity showing different selectivity and MICs for each microorganism. The highest content of bioactive compounds in the first stages might account for the better results found in their antimicrobial activity. For *L. deliciosus* stage I (immature) presented strong antimicrobial activity, while in *L. piperatus*, the most effective stage was stage II (mature with immature spores). Until mature spores are not present, the results seem to be independent of maturity degree, relying on the bioactive compounds content present in each stage. Similar correlation between phenolics content and antimicrobial properties was already observed by us in a previous assay (see chapter 3.2.3).

L. piperatus showed better results than *L. deliciosus* in the same stage of maturity (lower MICs), which is in agreement with the higher content of bioactive compounds found in the first species (**table 3.4.10**). Nevertheless, there might be other antimicrobial compounds besides those determined in this study.

Table 3.4.11. Antimicrobial activity of the mushrooms in different stages of maturity.

	MIC (mg/mL)							
Samples	*B. cereus*	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*E. coli*	*K. peumoniae*	*C. albicans*	*C. neoformans*
L. deliciosus SI	0.5 (+++)	0.5 (+++)	5 (++)	5 (++++)	50 (-)	50 (-)	50 (-)	50 (-)
L. deliciosus SII	50 (-)	5 (+++)	50 (+++)	5 (++++)	50 (-)	50 (-)	50 (-)	50 (-)
L. deliciosus SIII	50 (-)	50 (-)	50 (-)	50 (-)	50 (-)	50 (-)	50 (-)	50 (-)
L. piperatus SI	50 (-)	50 (-)	0.5 (++++)	5 (++++)	50 (-)	5 (++)	50 (-)	50 (-)
L. piperatus SII	0.5 (++++)	0.5 (++++)	0.5 (++++)	0.5 (+++)	5 (+++)	0.5 (++)	50 (-)	50 (-)
L. piperatus SIII	50 (-)	50 (-)	50 (-)	50 (-)	50 (-)	50 (-)	50 (-)	50 (-)
Ampicillin	0.00313 (++++)	0.0125 (++++)	0.00625 (++++)	0.00625 (++++)	0.00625 (++++)	0.00625 (++++)	NT	NT
Cycloheximide	NT	NT	NT	NT	NT	NT	0.0125 (++)	0.00625 (++++)

No antimicrobial activity at the higher tested concentration (-), inhibition zone < 1 mm. Slight antimicrobial activity (+), inhibition zone 2-3 mm. Moderate antimicrobial activity (++), inhibition zone 4-5 mm. High antimicrobial activity (+++), inhibition zone 6-9 mm. Strong antimicrobial activity (++++), inhibition zone > 9 mm. Standard deviation ± 0.5 mm. NT, not tested.

Fruiting bodies of pungent *Russulaceae* species (genera *Lactarius* and *Russula*) initially contains a biologically inactive fatty acid ester of velutinal (stearoylvelutinal). Upon injury (by insects or snails, biting and chewing the fruitbody) that compound is enzymatically transformed within seconds to pungent unsaturated dialdehydes and related sesquiterpenes. For instance, *L. piperatus* form velleral and piperdial. The mechanism of these conversions seems to constitute a chemical defense system that protects the fruit bodies of these species against parasites and fungivors (Jonassohn, 1996).

The extracts inhibited all the tested Gram + bacteria, showing no antifungal activity. Concerning Gram – bacteria, only *L. piperatus* revealed activity against the three species tested, being *E. coli* and *K. pneumoniae* resistant to *L. deliciosus* in all stages of maturity. As expected due to its lower content in bioactive compounds, the mature stage with mature spores (stage III) was the less effective, presenting no antimicrobial activity at 50 mg/mL. Usually, pure active compounds reveal more activity than crude extracts and, as expected, the standards ampicillin (antibacterial) and cycloheximide (antifungal) presented lower MICs than the mushrooms extracts.

Antimicrobial compounds with more or less strong activities could be isolated from many mushrooms and they could be benefit for human. Till now, only compounds from microscopic fungi are on the market as antibiotics and, therefore, it is important to screen mushrooms antimicrobial activity and conclude about the best stage of maturity to harvest wild species. The knowledge about their chemical composition in different stages of fruiting body maturity will be also useful in order to find the best stage to achieve better functional and nutritional properties. In this study we concluded that the last stage of fruiting body maturity is not recommended for those proposals.

CHAPTER IV

CONCLUSIONS

4.1. Conclusions

This investigation provided an insight to the chemical composition and bioactive properties of nineteen wild mushroom species (*Agaricus arvensis*, *Agaricus bisporus*, *Agaricus romagnesii*, *Agaricus silvaticus*, *Agaricus silvicola*, *Cantharellus cibarius*, *Hypholoma fasciculare*, *Lactarius deliciosus*, *Lactarius piperatus*, *Lepista nuda*, *Leucopaxillus giganteus*, *Lycoperdon molle*, *Lycoperdon perlatum*, *Macrolepiota mastoidea*, *Macrolepiota procera*, *Ramaria botrytis*, *Sarcodon imbricatus*, *Tricholoma acerbum* and *Tricholoma portentosum*) collected in the region of Bragança, Northeast of Portugal, in order to valorise these products as sources of nutrients and nutraceuticals.

The analysis of nutrients included determination of proteins, fats, ash, and carbohydrates. The macronutrient profile in general revealed that the wild mushrooms were rich sources of protein and carbohydrates, and had low amounts of fat. On the basis of the proximate analysis, it can be calculated that an edible portion of 100 g of fresh mushrooms assures, on average, 30 Kcal (127 KJ). The composition in individual sugars was also determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI), being mannitol and trehalose the most abundant sugars. The analysis of fatty acid composition was performed by gas chromatography coupled to a flame ionization detector (GC-FID) and allowed the quantification of twenty three fatty acids. Unsaturated fatty acids (UFA) and, in particular oleic and linoleic acids, were predominant.

R. botrytis revealed the highest energetic value, but with the highest protein content and lowest fat content. It also presented the highest MUFA and the lowest SFA levels, which may be relevant since the substitution of SFA with MUFA leads to an increase in HDL cholesterol and decrease in LDL cholesterol, triacylglycerol, lipid oxidation, and LDL susceptibility to oxidation. Concerning sugar composition this species presents the highest percentage of mannitol, which functions to provide support and expansion of the fruit body as other sugar alcohols. *Ramaria botrytis* is one of the largest of the coral fungi and is considered an excellent edible by some mycophagists.

It was also observed that proteins content and MUFA and PUFA percentages increased with the fruiting body maturity stage, while carbohydrates and SFA contents decreased.

The maturity stage did not significantly affect the individual sugar profile. Cooked samples proved to have lower nutrients concentrations than dried or frozen samples. In what concerns to fatty acids and sugar individual profiles, only cooking proved to be relevant, the cooked samples presenting higher MUFA, and lower PUFA and sugars contents.

The chemical composition and energy values of the Portuguese wild edible mushrooms clearly indicate that they provide key nutrients such as protein and carbohydrates. Being a relatively good source of protein and carbohydrate, they prove to be excellent foods that can be used in low caloric diets for their low contents of fat and energy. Nevertheless, the high nutritional quality and unique flavours of these mushrooms are likely to be lost if these wild edibles are not documented. Therefore, it seems imperative that a nutritional database of them is set up to retain the information on these unique species and for a better management and conservation of this natural resource and habitats related to them.

The analysis of nutraceuticals included determination of phenolic compounds including phenolic acids, total polyphenols and flavonoids, tocopherols, ascorbic acid and carotenoids. Phenolic compounds were determined in a first approach by the Folin-Ciocalteu assay, and then by high-performance liquid chromatography coupled to photodiode array detector and mass spectrometer (HPLC-DAD-ESI/MS). Four phenolic acids (protocatechuic, *p*-hydroxybenzoic acid, *p*-coumaric and cinnamic acid) and two vanillic acid isomers were detected, identified and quantified. *p*-Hydroxybenzoic acid was found in the majority of the samples. *R. botrytis* showed the highest phenolic acids concentration (356.7 mg/Kg, dry matter) due to the significant contribution of protocatechuic acid. No flavonoids or other phenolic families were found in the samples analysed, indicating that phenolic acids were the characteristic phenolics in the mushrooms, and that they may be contributing to the antioxidant properties of these products. Besides their importance as antioxidants for the human health, the phenolic acids analysis may also be useful in taxonomic studies involving mushroom species,.

The determination of tocopherols in mushrooms was also performed by normal-phase high-performance liquid chromatography (NP-HPLC). For the analysis of these compounds a new extraction methodology was described without saponification step, and new chromatographic conditions including fluorescence detection, and simultaneous detection of α and β-tocopherol isomers were optimised. The results

obtained demonstrate that the proposed method is sensitive, precise, and accurate allowing the determination of tocopherols. Individual tocopherol profiles revealed β-tocopherol as the major compound. α-Tocopherol was found in all the species with exception of *A. romagnesii*, while δ-tocopherols were not detected in any of the samples. *A. silvaticus* presented the higher content of tocopherols (3,232 ng/g) while *Cantharellus cibarius* revealed the lower content (213.5 ng/g).

The analysed mushrooms contain other useful phytochemicals such as ascorbic acid, β-carotene and lycopene. The analysed mushrooms contain other useful phytochemicals such as ascorbic acid, β-carotene and lycopene. Among the phytochemicals analysed, the highest levels corresponded to phenolic compounds (~293 – 6773 mg/Kg, dry matter), followed by ascorbic acid (~7 – 290 mg/Kg, dry matter), carotenoids (~0.5 – 44 mg/Kg, dry matter) and tocopherols (~0.2 – 11 mg/Kg, dry matter).

The antioxidant activity of all the mushroom species was screened through chemical and biochemical techniques. The chemical assays consisted of evaluation of their reducing power, radical scavenging activity and β-carotene bleaching inhibition, while biochemical assays evaluated the lipid peroxidation inhibition capacity, using erythrocytes and brain cells as models.

All the species proved to have antioxidant activity but *R. botrytis* proved to be the most active species, presenting lower EC_{50} values (less than 1 mg/mL) in all the antioxidant activity assays. The higher content in phenolic compounds might account for the better antioxidant properties found in this species.

The effects of fruiting body maturity on antioxidant activity and antioxidants production were evaluated. The highest antioxidant contents and the lowest EC_{50} values for antioxidant activity were obtained in the mature stage with immature spores.

The effects of processing and cooking practices on the antioxidant activity were also investigated. Cooked samples proved to have lower antioxidant activity than dried or frozen samples.

We reported the first approach to the antioxidant potential evaluation of the edible mushroom *Leucopaxillus giganteus* mycelium obtained in the presence of four different carbon and nitrogen sources. Despite the use of *Leucopaxillus* mushroom species in chemical industry for extraction of clitocybin antibiotic, the production of its mycelium for pharmacological applications has not been explored. The antioxidants concentration

increased along the growth time as a response to the oxidative stress and, therefore, free radicals production. The aldohexose glucose and diammonium phosphate proved to be the most appropriate carbon and nitrogen sources to increase antioxidant activity, leading to the highest content in phenolics and lowest EC_{50} values. Significantly negative linear regressions were established between total phenolic compounds contents and antioxidant activity, which support that the extracts mechanism of action for the different antioxidant activity assays (reducing power, DPPH scavenging activity, β-carotene-bleaching inhibition, erythrocyte hemolysis inhibition, and lipid peroxidation inhibition measured by thiobarbituric acid reactive substances formation) may be identical, being related with the content in those compounds and their free radical scavenging activity.

The antimicrobial properties of the mushrooms were screened against fungi (*Candida albicans*, *Cryptococcus neoformans*), Gram-positive (*Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*) bacteria. The growth of Gram-positive bacteria was well inhibited by the majority of the mushrooms, while Gram-negative bacteria and fungi were more resistant. Mature fruiting bodies with mature spores presented lower antimicrobial activity, which was in agreement with the bioactive compounds contents found in those samples.
Some mushrooms extracts inhibited medically important microorganisms with very low MIC (minimal inhibitory concentration), in some cases, even lower than the standards. This suggests that they are potential sources of new antimicrobial agents. With an increasing number of bacteria developing resistance to commercial antibiotics, such as MSRA (methicillin-resistant *S. aureus* and *Pseudomonas*), extracts and derivatives from mushrooms hold great promise for novel medicines.

Overall, the analyzed mushrooms contain very useful nutraceuticals such as UFA, phenolic compounds, tocopherols, ascorbic acid, and carotenoids which could be extracted for the purpose of being used as functional ingredients namely against microbial infections or chronic diseases related to oxidative stress. Public health authorities consider prevention with nutraceuticals as a powerful instrument in maintaining and promoting health, longevity and life quality. The beneficial effects of nutraceuticals will undoubtedly have an impact on nutritional therapy; they also

represent a growing segment of today's food industry. Thus, these mushrooms might be used directly in diet and promote health, taking advantage of the additive and synergistic effects of all the bioactive compounds present. The combination of their bioactive compounds and rich nutritional composition (high contents in protein and carbohydrates, low contents in fat with the precious contribution of unsaturated fatty acids, and absence of *trans* fatty acids) makes them very special.

Over two-thirds of cancer-related death could be prevented through lifestyle modification, particularly through dietary means and, mushrooms consumption, might contribute to minimize cancer risks trough antioxidants input. Also, antimicrobial compounds with more or less strong activities could be isolated from some mushrooms that could be benefit for humans. Till now, only compounds from microscopic fungi are on the market as antibiotics and, therefore, it is important to screen mushrooms antimicrobial activity and conclude about the best stage of maturity to harvest species. The knowledge about their chemical composition in different stages of fruiting body maturity will be also useful in order to find the best stage to achieve better functional and nutritional properties; we concluded that the last stage of fruiting body maturity is not recommended for those proposals. Not only mushrooms but also their mycelia may be a good candidate for employment as antimicrobial agent against bacteria responsible for human gastrointestinal and respiratory tract infections. Mushrooms mycelia may also constitute a good source of healthy compounds, namely phenolic compounds, suggesting that they could be useful in the prevention of diseases in which free radicals are implicated.

Finally, the findings from this investigation do not only contribute for a better knowledge of wild mushrooms but also to their valorisation, in a commercial aspect, due to the great intensity of mushrooms harvest in the Northeast of Portugal, and also as possible sources of phytochemicals/nutraceuticals for the pharmaceutical and food industries.

4.2. Conclusiones

Los estudios realizados han proporcionado un conocimiento profundo en cuanto a composición química y propiedades bioactivas de diecinueve especies de setas silvestres (*Agaricus arvensis, Agaricus bisporus, Agaricus romagnesii, Agaricus silvaticus, Agaricus silvicola, Cantharellus cibarius, Hypholoma fasciculare, Lactarius deliciosus, Lactarius piperatus, Lepista nuda, Leucopaxillus giganteus, Lycoperdon molle, Lycoperdon perlatum, Macrolepiota mastoidea, Macrolepiota procera, Ramaria botrytis, Sarcodon imbricatus, Tricholoma acerbum* y *Tricholoma portentosum*), valorizando los mismos como fuentes de nutrientes y nutracéuticos.

El análisis de nutrientes incluyó la determinación de proteínas, grasas, cenizas, y carbohidratos. El perfil de macronutrientes, en general, reveló que los hongos silvestres son fuentes ricas de proteínas e hidratos de carbono y que poseen poca cantidad de grasa. Se puede calcular que una porción comestible de 100 gramos de estos hongos aporta, en promedio, 30 Kcal (127 KJ).

La composición en los distintos azúcares se determinó por cromatografía líquida de alta resolución acoplada a un detector de índice de refracción (HPLC-RI), poniendo de manifiesto que manitol y trehalosa eran los azúcares más abundantes. El análisis de composición de ácidos grasos se realizó por cromatografía de gases acoplada a un detector de ionización de llama (GC-FID) y permitió la cuantificación de veintitrés ácidos grasos. Los ácidos grasos insaturados (UFA) eran predominantes y en particular, los ácidos oleico y linoleico.

Ramaria botrytis era la especie con el más alto valor energético, aunque debido a su mayor contenido de proteínas, presentando menor contenido de materia grasa. También era la especie que poseía los mayore niveles de ácidos grasos monoinsaturados (MUFA) y los valores más bajos de saturados (SFA). Esta circunstancia puede ser relevante, ya que es conocido que la sustitución de SFA por MUFA conduce a un aumento en los niveles de colesterol-HDL y disminución en los de colesterol-LDL, triglicéridos, tasa de oxidación lipídica y susceptibilidad de LDL a la oxidación. En cuanto a la composición de azúcares, esta especie presentaba el mayor porcentaje de manitol, que proporciona apoyo a la expansión del cuerpo frutificante al igual que otros azúcares-alcohol. *Ramaria botrytis* es una de las especies más representativa de hongos con forma de

coral y es considerada por algunos micologistas excelente desde el punto de vista comestible.

Otros aspectos observados fueron que los contenidos de proteínas y los porcentajes de MUFA y PUFA se elevan con la maduración del cuerpo fructificante, mientras que disminuye el contenido de hidratos de carbono y el porcentaje de SFA. Además, el grado de madurez no afecta significativamente el perfil individual de azúcares. En lo relativo al procesado, las muestras cocinadas mostraban menor concentración de nutrientes que las secas o congeladas. Respecto a perfiles de ácidos grasos y de azúcares individuales, sólo las muestras cocinadas mostraban alguna diferencia, al presentar porcentajes superiores de MUFA e inferiores de PUFA y azúcares.

En conclusión, se pudo establecer que los hongos silvestres portugueses comestibles son una buena fuente de proteínas e hidratos de carbono y que poseen bajos contenidos de grasa y energía, por lo que resultan adecuados para dietas bajas calorías. Sin embargo, la alta calidad nutricional y sabor único de estos hongos silvestres es probable que se pierda si los mismos no están documentados. Por lo tanto, es imprescindible configurar una base de datos nutricionales de los mismos para retener la información sobre estas especies únicas y para una mejor gestión y conservación de este recurso natural y los hábitats relacionados con las mismas.

El análisis de nutracéuticos incluyó la determinación de compuestos fenólicos, tocoferoles, ácido ascórbico y carotenoides. Se determinaron los compuestos fenólicos totales con el método de Folin-Ciocalteu y también se realizó un análisis individual por cromatografía líquida de alta resolución acoplada a detector de fotodiodos y espectrometría de masas (HPLC-DAD-ESI/MS). Cuatro ácidos fenólicos (ácido protocatéquico, p-hidroxibenzoico, *p*-cumárico y cinámico) y dos isómeros del ácido vaníllico fueron detectados y cuantificados. El ácido *p*-hidroxibenzoico era el compuesto más habitualmente presente en las muestras de hongos estudiadas. *Ramaria botrytis* mostró la más alta concentración de ácidos fenólicos (356,7 mg / Kg, materia seca), debido a la importante contribución de ácido protocatéquico. No se encontraron flavonoides ni otras familias de compuestos fenólicos, sugiriendo que los ácidos fenólicos son los compuestos fenólicos característicos de las setas y los que deben estar contribuyendo a sus propiedades antioxidantes. Estos ácidos podrían, por otra parte, ser útiles en estudios taxonómicos para la clasificación de especies de hongos.

La determinación de tocoferoles se realizó por cromatografía líquida de alta resolución en fase normal (HPLC-NP). Para ello se puso a punto una nueva metodología de extracción sin el paso de saponificación, y se optimizaron también nuevas condiciones, para el análisis cromatográfico, empleando un detector de fluorescencia, que permitían la detección simultánea de los isómeros α y β-tocoferol, habitualmente mal separados en otros métodos descritos en la bibliografía. El método propuesto fue sometido a ensayos de validación, que demostraron que era sensible y preciso. El perfil individual de tocoferoles reveló que el β-tocoferol era el principal compuesto en las setas estudiadas. El α-tocoferol se encontraba en todas las especies con excepción de *A. romagnesii*, mientras que no se detectó δ-tocoferol en ninguna de las muestras. *A. silvaticus* presentaba el mayor contenido de tocoferoles (3.232 ng/g), mientras que *Cantharellus cibarius* reveló el contenido más bajo (213,5 ng/g).

Las setas analizadas contenían, además, otros fitoquímicos, como ácido ascórbico, β-caroteno y licopeno. Entre los fitoquímicos encontrados, los niveles más elevados correspondían a los compuestos fenólicos (~293 – 6773 mg/Kg, sobre materia seca), seguidos de ácido ascórbico (~7 – 290 mg/Kg, sobre materia seca), carotenoides (~0.5 – 44 mg/Kg, sobre materia seca) y tocoferoles (~0.2 – 11 mg/Kg, sobre materia seca)

La actividad antioxidante de todas las especies de setas se valoró mediante métodos químicos y bioquímicos. Los ensayos químicos consistieron en la evaluación del poder reductor, la inhibición del radical libre DPPH y la inhibición del blanqueamiento del β-caroteno, mientras que en los ensayos bioquímicos se evaluó la capacidad de inhibición de la peroxidación lipídica, utilizando dos sistemas modelo: eritrocitos y células de cerebro.

Todas las especies demostraron actividad antioxidante, pero *R. botrytis* resultó ser la especies más activa con valores más bajos de EC_{50} (inferiores a 1 mg/mL) en todos los ensayos de actividad antioxidante. El mayor contenido en compuestos fenólicos podría explicar las mejores propiedades antioxidantes encontradas en esta especie.

Fue también evaluada la influencia de la maduración del cuerpo fructificante sobre la actividad antioxidante y la producción de fitoquímicos. Los contenidos más altos de antioxidantes y los valores más bajos de EC_{50} en los ensayos de actividad antioxidante se obtuvieron en la fase de cueropo maduro con esporas inmaduras. También se estudió el efecto del procesado y prácticas culinarias sobre la actividad antioxidante, encontrando que las muestras cocinadas poseían menor actividad antioxidante que las

secas o congeladas. Asimismo, se evaluó el potencial antioxidante del micelio de la seta comestible *Leucopaxillus giganteus* en función de la fuente de carbono y de nitrógeno empleada para su crecimiento, ya que a pesar de la utilización de especies de *Leucopaxillus* en la industria química para la extracción del antibiótico clitocibina, la producción de su micelio para aplicaciones farmacológicas no había sido aún explorada. Se observó que la concentración de antioxidantes aumentaba a lo largo del tiempo de crecimiento del micelio, posiblemente como respuesta al estrés oxidativo y, por tanto, a la producción de radicales libres. La aldohexosa glucosa y el fosfato diamónico demostraron ser las fuentes de carbono y nitrógeno más apropiadas para aumentar la actividad antioxidante del micelio, dando lugar al contenido más alto de compuestos fenólicos y a valores de EC_{50} más bajos. Se encontró una relación entre los contenidos de compuestos fenólicos totales y actividad antioxidante de las muestras, en los distintos ensayos empleados (poder reductor, inhibición del radical DPPH, inhibición del blanqueamiento del β-caroteno, inhibición de la hemólisis eritrocitaria e inhibición de la peroxidación), lo que apunta a la existencia de un mismo mecanismo de acción relacionado con la capacidad de estos compuestos para inhibir radicales libres.

Se examinaron también las propiedades antimicrobianas de las setas frente a hongos (*Candida albicans*, *Cryptococcus neoformans*), bacterias Gram-positivas (*Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*) y Gram-negativas (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*). La mayoría de las setas inhibían el crecimiento de las bacterias Gram-positivas, mientras que bacterias Gram-negativas y hongos eran más resistentes. El cuerpo fructificante presentaba menor actividad antimicrobiana en la fase de esporas maduras que de inmaduras, lo que estaba de acuerdo con el contenido de compuestos bioactivos encontrados en cada una de estas etapas.

Algunos de los extractos ensyados fueron capacesde inhibir el crecimiento de microorganismos con importancia medicinal, presentando concentraciones mínimas inhibitorias muy bajas y, en algunos casos, inferiores incluso a las de las sustancias antimicrobianas empleadas como referencia. Con un número cada vez mayor de bacterias resistentes a antibióticos comerciales, como las cepas meticilina-resistentes de *S. aureus* y *Pseudomonas* (MSRA), este tipo de productos aparecen, de este modo, como fuentes potenciales para la obtención de nuevos agentes antimicrobianos de posible interés clínico.

De manera general, se puede concluir que las setas analizadas contienen nutracéuticos de gran interés como ácidos grasos insaturados, compuestos fenólicos, tocoferoles, ácido ascórbico o carotenoides, que podrían ser de gran utilidad para su empleo como ingredientes funcionales, en la prevención de enfermedades crónicas relacionadas con el estrés oxidativo. Además, estos hongos pueden ser utilizados de manera directa como alimentos. La combinación de sus compuestos bioactivos, con posibles efectos aditivos o sinérgicos, y su adecuada composición nutricional (alto contenido en proteínas e hidratos de carbono, bajo contenido en grasa, con el valioso aporte de ácidos grasos insaturados y la ausencia de ácidos grasos *trans*) los hace alimentos especialmente valiosos. Hay que tener en cuenta que el aprovechamiento de los potenciales efectos beneficiosos de los nutracéuticos representa un segmento creciente de la actual industria alimentaria y tiene, sin duda, un impacto en la terapia nutricional, siendo considerados por las autoridades en materia de salud pública como un instrumento en las estrategias de mantenimiento y promoción de la salud, longevidad y calidad de vida. Se considera que en torno a dos tercios de los casos de cáncer podrían reducirse mediante una modificación de estilo de vida, en el cual juega un papel determinante la dieta y, en este sentido, el consumo de estos hongos podría representar una contribución interesante.

Por otra parte, los compuestos antimicrobianos, más o menos activos, que pueden ser obtenidos de estos hongos constituyen otro aspecto de potencial interés en terapéutica humana. Hasta ahora, sólo se encuentran en el mercado compuestos antibióticos producidos por hongos microscópicos; la presencia en las setas de compuestos con actividad antimicrobiana abre nuevas perspectivas que debería ser exploradas. Son necesarios más estudios para identificar estas sustancias y concluir sobre sus efectos, así como evaluar la mejor etapa en la maduración del cuerpo fructificante para la recolección de las setas, con vistas a conseguir los mayores contenidos de sustancias bioactivas. En el estudio aquí realizado, se ha encontrado que la última fase de crecimiento del cuerpo fructificante (maduro con esporas maduroas) no es la etapa más adecuada para obtener un producto con la mayor actividad, funcionalidad y propiedades nutricionales posibles.

Por último, se ha podido concluir que no sólo las setas, sino también su micelio resulta un buen candidato para el empleo en la producción de sustancias fitoquímicas y agentes

antimicrobianos, que podrían tener utilidad contra bacterias responsables de enfermedades gastrointestinales e infecciones de las vías respiratorias.

En definitiva, las conclusiones de esta investigación contribuyen a un mejor conocimiento de las setas silvestres, que debería repercutir positivamente en su valorización, tanto de cara a su comercialización directa, aspecto ya de gran importancia actual en el Nordeste de Portugal, como a la producción de fitoquímicos/nutracéuticos de gran interés para las industrias alimentaria y farmacéutica.

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APPENDIX 1