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Characterization of mushroom compounds and effect on neuronal ROS

Thesis Project in the scientific area of Chemical Engineering, supervised by Professors Doctors Rosa M. Quinta-Ferreira and M. Emília Quinta Ferreira and submitted to the Department of Chemical Engineering, Faculty of Science and Technology, University of Coimbra

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"Comece fazendo o que é necessário,

depois o que é possível e de repente,

você estará fazendo o impossível."

São Francisco de Assis

"Sê todo em cada coisa. Põe quanto és

No mínimo que fazes."

Ricardo Reis - Heterónimo de Fernando Pessoa

Dedico esta Tese de Mestrado,

aos meus pais,

Jorge** e **Maria João,

e ao meu irmão,

***José Ricardo**,*

por todo o amor e carinho.

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Resumo

Os cogumelos têm vindo a ser incluídos de forma cada vez mais frequente na alimentação humana, assumindo um papel importante, quer devido às suas características nutricionais e organoléticas, quer devido às diferentes formas de consumo, despertando sensações únicas, devido ao seu aroma e sabor que colocam e despertam em cada prato. Por outro lado, são também muito consumidos, devido às suas propriedades medicinais extraordinárias, sendo cada vez mais alvo de muitos estudos realizados neste âmbito. Estudos científicos e médicos demonstram as propriedades medicinais dos compostos extraídos de cogumelos para a prevenção das doenças mais diversas, sendo cada vez mais frequentes na prevenção e tratamento do cancro (Zaidman *et al*., 2005; Lemieszek *et al.*, 2013).

O objetivo deste trabalho consiste na caracterização química de quatro espécies de cogumelos, *Boletus edulis, Tricholoma equestre*, *Ganoderma lucidum* e *Ganoderma lingzhi* e no estudo do efeito de um dos seus compostos na atividade neuronal.

Neste estudo, investigou-se a ação de polissacarídeos extraídos dos cogumelos na atividade antioxidante de uma zona sináptica. Os experimentos consistem na adição de diferentes concentrações de tais extratos na região CA3 do hipocampo do cérebro no sistema sináptico de fibras musgosas usando fatias de cérebro de 400 μm de espessura.

Os resultados obtidos durante a caracterização química dos cogumelos permitiram verificar a existência de diferenças, em termos da sua composição em compostos fenólicos e polissacarídeos, quer ao nível qualitativo quer ano nível quantitativo. Este trabalho também permitiu tornar mais clara a distinção que deve existir no caso das espécies *Ganoderma lucidum* e *Ganoderma lingzhi*, pois apesar de pertencerem à mesma espécie, apresentam diferenças qualitativas e quantitativas nos compostos neles presentes.

Relativamente ao estudo do efeito dos polissacarídeos na formação de ROS em neurónios, foram testadas três concentrações diferentes: 0.1, 0.5 e 1 g/L, tendo-se registado uma maior diminuição da formação de espécies reactivas de oxigénio, quando se usaram concentrações destes compostos de 1 g / L. No entanto, tendo em conta o pequeno número de experiências realizado, não se pode garantir uma confiança nos resultados obtidos.

Palavras chave: cogumelos; ROS; *Boletus edulis*; *Tricholoma equestre*; *Ganoderma lucidum*; *Ganoderma lingzhi*;

Abstract

Mushrooms have been increasingly included in human food, taking on an important role, either due to their nutritional and organoleptic characteristics or due to the different forms of consumption, arousing unique sensations, due to their aroma and flavor put and wake up on each plate. On the other hand, they are also very consumed, due to their extraordinary medicinal properties, being more and more the target of many studies carried out in this scope. Scientific and medical studies demonstrate the medicinal properties of compounds extracted from mushrooms for the prevention of the most diverse diseases, being more and more frequent in the prevention and treatment of cancer (Zaidman *et al.*, 2005; Lemieszek *et al.*, 2013).

The aim of this work is the chemical characterization of four mushroom species, *Boletus edulis*, *Tricholoma equestre*, *Ganoderma lucidum* and *Ganoderma lingzhi*, and to study the effect of one of its compounds on neuronal activity.

In this study, we investigated the action of polysaccharides extracted from mushrooms on the antioxidant activity of a synaptic zone. The experiments consist of adding different concentrations of such extracts to the CA3 region of the brain hippocampus in the moss-synaptic system using 400 μm thick brain slices.

(....)

Keywords: mushrooms; ROS; *Boletus edulis*; *Tricholoma equestre*; *Ganoderma lucidum*; *Ganoderma lingzhi*;

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List of Acronyms and Abbreviations

ACSF	-	Artificial Cerebrospinal Fluid
AIR	-	Alcohol Insoluble Residue
CA1	-	Area 1 of the Ammon's horn *(cornu ammonis)*
CA2	-	Area 2 of the Ammon's horn *(cornu ammonis)*
CA3	-	Area 3 of the Ammon's horn *(cornu ammonis)*
CA4	-	Area 4 of the Ammon's horn *(cornu ammonis)*
CO_2	-	Carbon dioxide
DCF	-	2',7' - dichlorofluorescein
DNA	-	Deoxyribonucleic acid
GAE	-	Gallic Acid Equivalent
HPLC	-	High Performance Liquid Chromatography
H_2DCFDA	-	2`,7`- dichlorodihydrofluorescein diacetate
LDL	-	Low Density Lipoprotein
NAD(P)H	-	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
n.d.	-	no date
O_2	-	Oxygen
PUFAs	-	Polyunsaturated Fatty Acids
RNA	-	Ribonucleic acid
ROS	-	Reactive Oxygen Species
UV	-	Ultraviolet radiation
Vis	-	Visible radiation
WSB	-	Water Soluble Biopolymers
WSM	-	Water Soluble Material
$\lambda_{emission}$	-	Emission wavelength (nm)
$\lambda_{excitation}$	-	Excitation wavelength (nm)
MΩ.cm	-	Ultra pure water resistivity units
mM	-	millimolar
º C	-	degree Celsius

Glossary

Bioactive compouds	- Nutritional constituents, naturally present in small amounts in foods, which exhibit potent biological activity
Blade	- An element of the fruiting body, located underneath the hat, on the surface of which are the cells producing the spores.
CCD Camera	- Charge-coupled device camera. A CCD camera allows the acquisition of two-dimensional images of the preparation.
Cuticle	- Outer layer or film covering the outer surface of the mushroom hat
GABA	- Gamma-amino-butyric acid. An amino acid that is an inhibitory transmitter in many central synapses.
Gap junction	- The very close approximation of the presynaptic and possynaptic membranes that characterizes electrically transmitting synapses.
Glutamate	- An excitatory transmitter derived from the diacidic amino acid, glutamic acid.
KeV	- kiloelectron volt. A unit of energy approximately equal to 1.6×10^{-19} joules that corresponds to the amount of energy acquired (or lost) by the charge of a single electron moving through an electric potential difference of one volt.
Rhabdomyolysis	- Degenerative muscle disease, which causes destruction of skeletal muscle cells, resulting in damage to the kidneys and cardiac muscle, and latter case, death.
Spore	- A specialized element for the propagation of fungi, able to withstand adverse conditions and germinate in a favorable environment.
Stipe	- A structure that support the cap of a mushroom.
Limbic system	- A set of primitive structures, under the cerebral cortex, that is responsible for some of the most basic human instincts and behaviors.
Xenobiotic	- Substance found in biological systems, but of external origin.

I. Introduction

Today, due to the prolongation of the average life expectancy, as well as the increase of the aggressions suffered from the surrounding environment, there is an increasing incidence of diseases around the world.

Thus, more and more people in the 21st century suffer from neuronal diseases such as Alzheimer's and Parkinson's, depression, premature aging, cancer, and despite the evolution of science, it has not yet been possible to explain why they occur so early.

It is believed that the increase of these pathologies is related to failure in the equilibrium (homeostasis) of reactive oxygen species (ROS) in the central nervous system. This imbalance is essentially due to the adopted lifestyle, being commonly associated with situations that cause great worry and stress, and with the increase of the aggressions suffered from the surrounding environment, in which many chemical compounds harmful to health are present.

The main motivation of the present study was the desire to make a positive contribution to improving the quality of life of the population with regard to health issues. It was therefore intended to characterize the chemical composition of mushrooms and the effect of mushroom phenolic compounds, on synaptic ROS signals.

For this propose, the mushrooms *Boletus edulis*, *Tricholoma equestre*, *Ganoderma lucidum* and *Ganoderma lingzhi* were studied. The main focus was then the chemical characterization of these mushrooms, particularly in terms of their composition in phenolic compounds, monosaccharides and chemical elements that form part of their fruiting body.

In addition, the effect of the polyssacharides extracted from *Boletus edulis* on synaptic ROS evaluated, more specifically on the mossy fiber synapses from hippocampal CA3 area, using a fluorescent ROS indicator.

In the brain, the CA3 region has attracted much attention in the last years because it plays a key role in memory processes and neurodegenerations (Cherubini, E. and Miles, R., 2015).

For the accomplishment of these objectives, the chemical characterization took place at the Laboratory of Chemistry, of the Laboratory Blocks of the University of Trás-os-Montes e Alto Douro, in Vila Real. The neuronal experiments were carried out at the Biophysics Laboratory of the Physics Department of the University of Coimbra.

For a better understanding of the subjects that are dealt with in this work the thesis includes, at the beginning, some concepts that are considered fundamental.

Thus, *Chapter II - State of the art* - begins with a brief presentation of the hippocampal area and of synaptic transmissions. Next, autofluorescence is described and, finally, the origin of reactive oxygen species is presented, as well as the properties of the fluorescent indicator that that was used to detect some of them.

Afterwards, the importance of both endogenous and exogenous antioxidants in the human body, particularly in the brain, is addressed. Also a bibliographical review was made of the various antioxidants compounds present in foods and of their effects on the human body.

Lastly, a brief characterization of the mushrooms *Ganoderma lucidum*, *Ganoderma lingzhi*, *Tricholoma equestre* and *Boletus edulis*, was made stressing their importance mainly in the medicinal and nutritional areas.

The experimental procedures used in order to achieve the above mentioned objectives is presented in *Chapter III - Materials and methods*, being the results shown and their discussion made, in *Chapter IV - Results and discussion*.

Finally, *Chapter V - Conclusions and future work perspectives* - presents a synthesis of the main conclusions that have been reached. Being this work necessarily limited in time, several questions remain unanswered and others were not even addressed.

Thus, the thesis ends with the presentation of some suggestions for future research work considered of interest.

II. State of the art

II. 1. Hippocampal areas

The brain is the main organ and center of the nervous system, being one of the most complex of the human organism. The nervous system is responsible for directing all our activities, acting on the remaining organs of the body, namely, in the functioning of internal organs, movements, thoughts, emotions (The Reader's Digest Association, Inc., 1987).

One of the main components of the brain is the hippocampus, which has a bilateral symmetry and is located under the cerebral cortex being part of the limbic system. This cerebral area plays a key role with regard to spatial memory and orientation. In addition, the hippocampus that has a shape that resembles a seahorse, is one of the first zones to be affected in case of Alzheimer's disease, which causes problems of memory and disorientation (The Reader's Digest Association, Inc., 2017).

On the other hand, it is known that depression, slows down the processing power of the brain, affects the concentration and causes severe memory problems. There are even studies that reveal that people suffering from depression have physical changes in the brain itself. Researchers at King's College in London observed in magnetic resonance imaging studies of the brain, during episodes of acute depression, that the hippocampus had decreased in volume when compared to people without depression (The Reader's Digest Association, Inc., 2017).

The term "hippocampus" is commonly used to describe two regions joined together: the dentate gyrus (*fascia dentata*) and the hippocampus proper (Ammon's horn). Both are composed of two main types of cells: granule cells ofthe dentate gyrus and pyramidal cells of *Cornu Ammonis* (CA) or Ammon's horn. The hippocampus is composed of four sub-domains, anatomically and functionally differentiated, named CA1, CA2, CA3 and CA4 (Andersen *et al.*, 2007; Cherubini and Miles, 2015). Each of these regions maintains an organized pattern of intrinsic and extrinsic connections, where the primary afferent to the hippocampus originates in the entorhinal cortex.

Figure 1 represents an hippocampal slice where the different hippocampal areas can be identified.

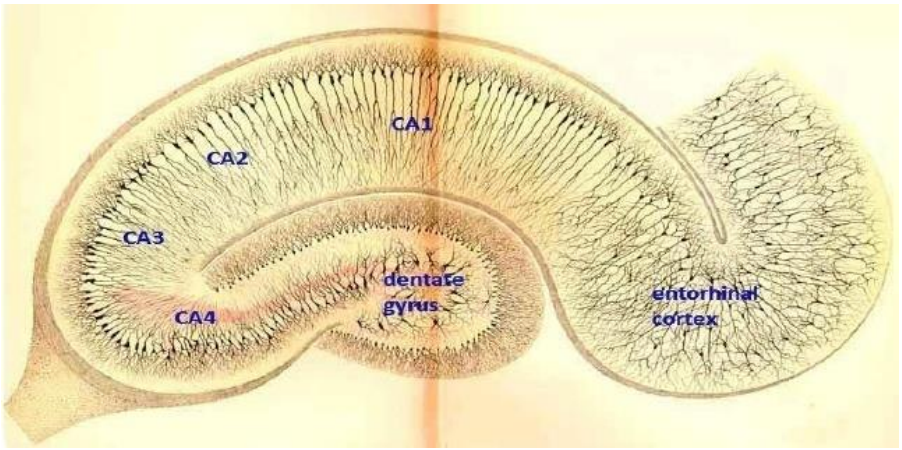


Figure 1 - Transverse hippocampal slice. Transverse slice of the hippocampus, with the different structures: dentate gyrus, Ammon's horn (divided into four distinct areas (CA1 to CA4)) and the entorhinal cortex (Adapted from https://pt.slideshare.net/amandahessborzacchini/hippocampus-13053807/11. Accessed March 2017).

According to Andersen *et al.*(2007), the entorhinal cortex can be considered to form part of the major hippocampal input pathway since cells from the entorhinal cortex give rise to axons that project, among other destinations, to the dentate gyrus. Bearing in mind that this is a unidirectional route, likewise, the principal cells of the dentate gyrus, the granule cells, give rise to axons called mossy fibers that connect with pyramidal cells of the CA3 field of the hippocampus ("mossy fiber - CA3 synapses"). The pyramidal cells of CA3, in turn, are the source of the major input to the CA1 hippocampal field (the Schaffer collateral axons). Finally, following the pattern of its predecessors, neurons in the CA1 region send their axons not only to the entorhinal cortex but also to the other regions of the brain (*subiculum*), being the main way out of the hippocampus (Andersen *et al*., 2007).

In Figure 2, shows the anatomical location of the hippocampus in the rat brains.

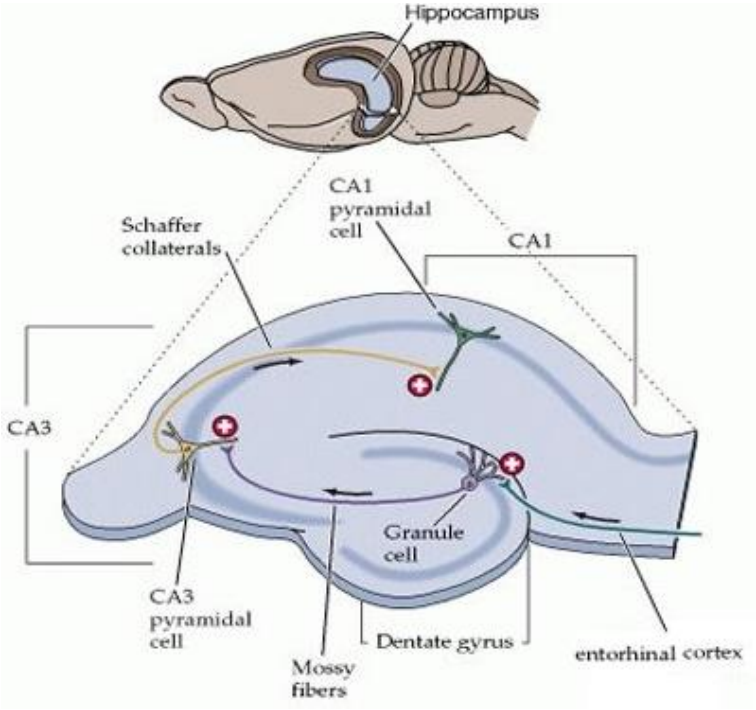


Figure 2 - The anatomical location of the hippocampus in the rat brains. (Taken from http://neurosciencenews.com/short-term-memories-created-by-researchers-in-vitro/. Accessed March 2017).

II. 2. Synaptic transmission

The central nervous system is characterized by the ability of cells to communicate and interact with each other, allowing them to receive and transmit signals to and from different parts of the body. The communication occurs through synapses that allow signals to be sent in an absolutely fast and precise way to other neurons or to other non-neuronal cells, such as the muscle fibers. It is through them that the electric impulse, that carries information, is transmitted. Synapses can be of two types, electrical or chemical (Hennig, n.d.).

In electrical synapses, the passage of ions or small molecules from one cell to another occurs through intercellular channels, called gap junctions. In this way, the transmission of information occurs instantaneously from one cell to other with the direct transfer of electric current between the presynaptic and postsynaptic cells, being particularly useful when the speed and the precision in the transmission of the impulse are fundamental (Andersen *et al*., 2007; Hennig, n.d.).

The chemical synapses are the most used in signal transmission in the central nervous system of the human species. These involve the transmission of signaling molecules (chemical mediators) called neurotransmitters by a neuron (presynaptic) and their detection by an adjacent (postsynaptic) neuron. These two zones are separated by a region called the

synaptic cleft. Neurotransmitters are located inside vesicles, being released by exocytosis in the presynaptic zone, binding and activating the receptors that are located in the membrane of the postsynaptic neuron, causing a functional response of the post-synaptic cell. (Andersen, *et al.*, 2007; Hennig, n.d).

In Figure 3 it is possible to observe the main differences that exist between the two types of synaptic transmissions.

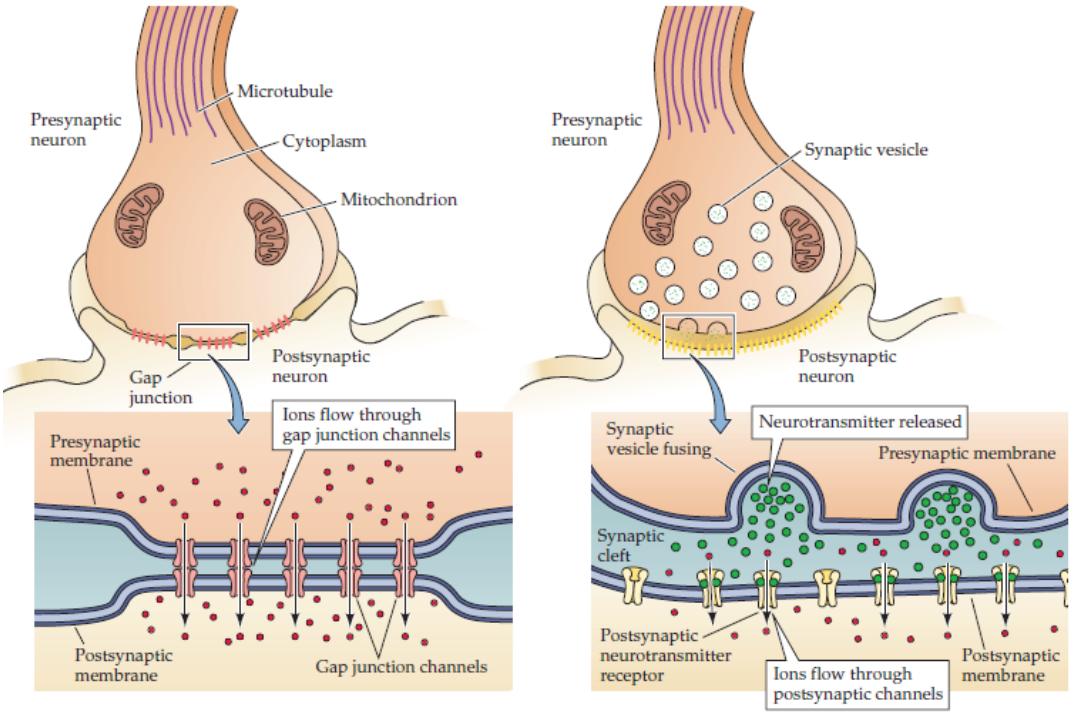


Figure 3 - Synaptic transmission. Schematic representation of an electrical synapse (left) and a chemical synapse (right) (Purves *et al.*, 2004).

There are two types of chemical synapses, excitatory and inhibitory, according to the type of neurotransmitter they release and how it affects the postsynaptic neuron. The most abundant excitatory neurotransmitter in the hippocampus is glutamate while the main inhibitory substance in chemical synapses is the neurotransmitter GABA (gamma-amino-butyric acid) (Andersen *et al.*, 2007).

Many substances interfere in cellular communication, affecting its normal functioning. For example, while under normal conditions glutamate is a neurotransmitter that activates neurons, in excess, it may be toxic to the brain and cause some of the degeneration of neurons that is responsible for Alzheimer's disease. There are also studies that suggest that depression affects the connections between neurons, allowing an amplification of the signals of thought as they pass from one cell to another, making it difficult to form and recover memories(The Reader's Digest Association, Inc., 2017).

II. 3. Autofluorescence

Fluorescence is a property that some substances possess after being excited with low wavelength radiation, resulting in a longer wavelength emission. Certain substances absorb the energy of ultraviolet light and emit radiation in the visible zone.

The fluorescence microscope it is widely used because it allows the study and observation of materials and of living cells, allowing, in this case, real-time information on the morphological and physiological state of cells and tissues. It is also useful for the detection of autofluorescence, which is due to the emission of molecules contained in the cells that become fluorescent when excited by wavelength radiation in the UV / Vis region. This fluorescence emission is an intrinsic property of cells to be distinguished from the fluorescent signals obtained by the addition of exogenous markers. Most of the phenomena of cellular autofluorescence originate in structures such as mitochondria and lysosomes (Monici, 2005). Autofluorescence can generally be detected in a fairly robust and stable manner using photomultiplier devices or CCD cameras, provided that measures are taken to minimize photobleaching and tissue damage due to excessive exposure to UV rays (Shuttleworth, 2010). The autofluorescence signals enable studies of mitochondrial function and dysfunction in brain slices, as well as providing robust maps of evoked postsynaptic neuronal activity (Shuttleworth, 2010).

Synaptic stimulation in brain slices is accompanied by changes in tissue autofluorescence, as they are a consequence of the metabolic changes occurring in the tissues. Autofluorescence excited by UV light is due to reduced NAD(P)H pyridine nucleotides. The pyridine coenzymes NAD(P)H, found in cells of all living organisms and used as "electron carriers" in metabolic oxidation-reduction reactions have a preponderant role in producing energy for the cell, and the ability to be autofluorescent. In particular, NAD(P)H imaging methods are very useful for monitoring the propagation of neuronal activity in the more complex brain tissues (Shuttleworth, 2010).

II. 4. Reactive Oxygen Species

Oxygen, O_2, is an abundant molecule in our environment, being indispensable to life, participating in many reactions of energy production in both animals and plants. Oxygen acts as the final acceptor of electrons, passing through several states of oxidation, leading to the

emergence of several reactive species, which have unpaired electrons and are capable of attacking any biomolecule.

O_2 undergoes partial reductions leading to the appearance of reactive oxygen species (ROS) which are oxygen-containing compounds and include free radicals such as superoxide anion (O_2•-), hydroxyl radical (HO•), hydroperoxy radical (HOO•), and species not radicals such as hydrogen peroxide (H_2O_2) and oxygen (O_2) (Birben *et al*., 2012).

The molecular oxygen in its ground state has two unpaired electrons in its outermost orbital. However, the two unpaired electrons have "spin" of the same orientation, thus making a less reactive O_2 molecule that react with only one electron. When O_2 accepts an electron, the superoxide anion ($O_2^{\bullet -}$) is formed, which is a precursor of most other reactive oxygen species (ROS). This reaction is not thermodynamically favorable and requires the addition of one more electron. In this way, the superoxide anion is reduced to hydrogen peroxide (H_2O_2) which, although not a radical, can generate the hydroxyl radical (HO•), as a result of further reduction. Finally, in the last step, with the addition of a new electron, the hydroxyl radical is reduced, giving rise to the water molecule (Bastos, 2014).

Figure 4 shows the steps necessary for generation of the reactive oxygen species.

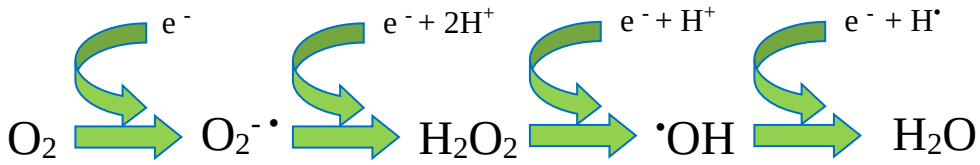


Figure 4 - The four steps to reduce O_2 progressively generate the reactive oxygen species (superoxide anion, hydrogen peroxide, hydroxyl radical and water).

These species are constantly produced by living organisms as the result of normal cellular metabolism. In addition, these products are also formed when the organism is exposed to various external agents such as ultraviolet light, ionizing radiation, xenobiotics, drugs, chemotherapeutics, as well as the inhalation of toxic chemicals present in environmental air and in the tobacco smoke (Smina *et al*., 2011; Kozarski *et al*., 2015).

In normal conditions, ROS are generated and eliminated constantly from the biological system, in order to ensure a balance in ROS levels. However, when the mechanisms of regulation do not work correctly, there may be an inbalance between the production and the elimination of ROS, causing a significant increase in the amount of ROS and thus, oxidative stress. This accumulation results in significant damage to cell structures, causing adverse changes in lipids, proteins and DNA, and in cellular functions. For these reasons excessive ROS formation is considered to be involved in a variety of disorders including cancer, neurological disorders, hypertension, diabetes and asthma (Birben *et al*., 2012).

II. 4.1. Fluorescent ROS indicator

Although a variety of chemicals exist, the detection of ROS in biological systems is difficult for a number of reasons, including method sensitivity and probe specificity (Hassani and Dupuy, 2013).

H_2DCFDA, a chemically reduced form of fluorescein, is widely used as an indicator of ROS, in living cells, namely in fluorescence microscopy, reacting with hydrogen peroxide and hydroxyl radicals (Hassani and Dupuy, 2013; H2DCFDA, H2-DCF,DCF).

2`,7`- dichlorodihydrofluorescein diacetate (H_2DCFDA) is an organic compound that has the chemical formula $C_{24}H_{16}Cl_2O_7$, as can be seen in Figure 5. (H2DCFDA (H2-DCF, DCF) Molecular Probes™).

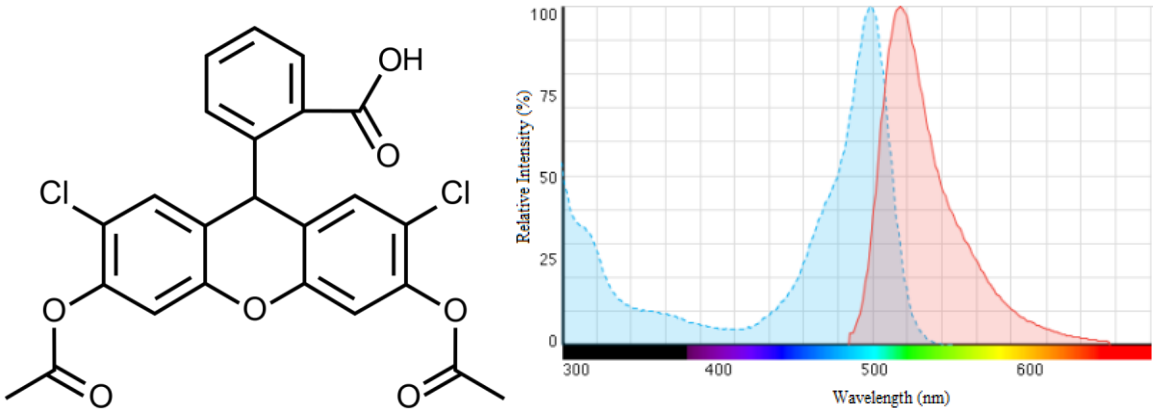


Figure 5 - The molecular structure (left) and the fluorescence spectrum (rigth) of the ROS indicator- H_2DCFDA. Excitation spectrum ($\lambda_{excitation}$ = 504 nm,blue); Emission spectrum ($\lambda_{emission}$ = 529 nm, red) (Adapted taken from H2DCFDA (H2-DCF, DCF) Molecular Probes ™).

It is a dye that is non-fluorescent when chemically reduced, however, after removal of the acetate groups by intracellular esterases and cell oxidation, it yields the highly green fluorescent compound, 2',7' - dichlorofluorescein (DCF) that is represented in Figure 6 (Biotium, H2DCFDA (2′,7′-Dichlorodihydrofluorescein diacetate; Thermofisher,H2DCFDA, H2-DCF, DCF)).

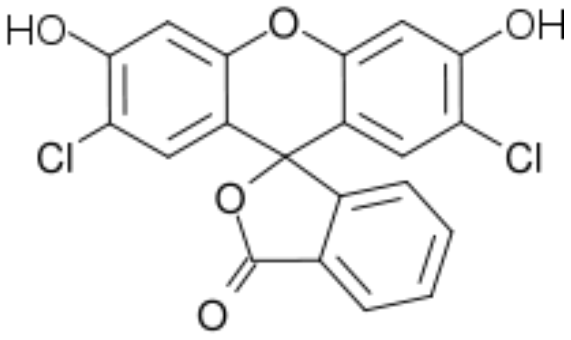


Figure 6 - The structure molecular of DCF.

II. 5. Antioxidants

The brain is particularly vulnerable to the effects of oxidative stress since it is a part of the body that has high energetic needs, that is, it consumes a lot of O_2 and therefore generates many free radicals that may damage healthy cells. In addition, given that brain tissue is rich in fat, polyunsaturated fatty acids (PUFAs), it becomes one of the targets most susceptible to the action of free radicals, since the free circulation of free radicals, "tears" the cell membranes, changes DNA, blocks synapses, and harms neural communication networks (Carper, 2014; Friedman, 2011).

However, humans and other organisms have evolved to develop several effective defense and repair mechanisms in order to protect themselves from the oxidative action of free radicals. The main defense mechanism against the production of ROS, is made by antioxidants.

Antioxidants are a heterogeneous set of endogenous or exogenous substances that protect cellular components from oxidative damage by blocking their damaging effect, thereby reducing the risk of potential mutations and carcinogen (Food Ingredients Brasil, 2016). Antioxidants can also be defined as aromatic compounds which contain at least one hydroxy group (Food Ingredients Brasil, 2015).

In the case of eukaryotic organisms, these have antioxidant enzymes, which comprise superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). These enzymatic antioxidants act as first-line defense agents in the direct elimination of ROS, reacting with oxidizing compounds and protecting cells and tissues from oxidative stress (Food Ingredients Brasil, 2009; Friedman, 2011). However, in some situations, these are insufficient to fully prevent oxidative damage, and an additional protection provided by nutritious and non-nutritive elements of food is critical for disease prevention, contributing to the defense of the organism and the maintenance of health (Reynertson *et al.*, 2008). Many foods contain antioxidants such as polyphenols, flavonoids, carotenoids (beta-carotene and lycopene), polysaccharides, terpenes, Vitamin C, Vitamin E, essential minerals such as zinc, selenium, iron, manganese, that act together to protect against ROS in sufficient ammouts to supply the needs of the body. Table 1 shows the effects of the compounds present in many foods, specially fruits and vegetables, in the body, with greater importance given to its antioxidant component.

Table 1 - Beneficial effects in the organism of some antioxidants components.

Components	Observations	References
Polyphenols	Polyphenols have a very important role in promoting health and preventing some diseases, playing a protective role against cardiovascular diseases, and interfering in the initiation and progression of cancer. In addition, they have anti-inflammatory, anti-aging, antiviral, antimicrobial actions.	(Robaszkiewicz *et al*., 2010)
	Other biological actions of polyphenols include an important role in free radical scavenging, in metal chelation enzyme modulation activities and in the reduction of the susceptibility of low density lipoproteins (LDL) to oxidation both in vitro and in vivo, an effect likely due to the property of these compounds to scavenge free radicals. On the other hand, polyphenols protect the brain, improving learning processes and memory.	(Rodrigo and Bosco, 2006)
Flavonoids	Flavonoids exhibit anti-allergic, anti-viral, anti-tumor, anti-inflammatory and vasodilatory effects in biological systems. In addition, they are known to act as antioxidants because of their role in suppressing free radical formation (either by inhibiting the activity of many enzymes or by the chelation of trace elements involved in the production) and by scavenging free radicals. They are also known to regulate and protect antioxidant defenses.	(Pietta, 2000)
Carotenoids	Several studies have revealed that increased consumption of carotenoid-rich foods is related to reduced risk of the onset of many neurodegenerative diseases, including various types of cancers, cardiovascular diseases and ophthalmological diseases. Preventive effects are	(Stahl and Sies, 2003)

	related to antioxidant activity, through sequestration and inactivation of free radicals, protecting cells and tissues from oxidative damage. In addition, carotenoids also influence cell signaling and may trigger redox-sensitive regulatory pathways.	
Beta-carotene	The beta-carotene activity in biological systems has been attributed to its ability to physically quench singlet oxygen.	(Di Mascio *et al.*, 1989)
Lycopene	Several in vitro studies have demonstrated the antioxidant capacity of lycopene. It is involved in various functions, including inhibition of cell proliferation and antioxidant potential capable of eliminating singlet oxygen and peroxyl radicals resulting from oxidative stress. Ingestion is associated with a reduction in the incidence of some types of cancers and the prevention of cardiovascular accidents.	(Bojórquez *et al.*, 2013)
Polysaccharides	Polysaccharides are know to have a protective effect against free radicals and reduce cell damage caused by mutagens.	(Boh *et al.*, 2007)
	Polysaccharides exhibit various activities, namely anticancer, immunostimulating, anti-blood coagulation, and antioxidants. It is also suggested that they are capable of enhancing the cell-mediated immune responses in vivo and in vitro and of acting as modifiers of the biological response.	(Wang *et al.*, 2013)
Triterpenes	Triterpenes are known to have a wide range of properties, including antimicrobial, antifungal, antiviral, antihyperglycemic, anti-inflammatory and antiparasitic, immunomodulatory properties, as well as possessing properties in cancer chemoprevention.	(Paduch *et al.*, 2007)

	Some triterpenes, such as, for example, oleanolic and ursolic acids, in small amounts, show a hepatoprotective effect, which may be due to their anti-oxidant and anti-inflammatory actions. It is further noted that these triterpenoids are also effective inducers of metallothionein which acts like glutathione in defending the body against many chemical insults that could induce liver injury.	
Vitamin C	Vitamin C provides protection against oxidative stress induced by cellular damage through the elimination of ROS.	(Traber and Stevens, 2011)
Vitamin E	Vitamin E (alpha-tocopherol) works as a fat-soluble antioxidant, eliminating hydroperoxyl radicals in lipid milieu. Thus, it has the ability to protect the membranes of nerve cells and tissues, which are composed primarily of fatty acids, from the damage caused by free radicals. It also protects fats in low density lipoprotein (LDL) from oxidation	(Traber and Stevens, 2011)
Zinc	Zinc plays an essential role in life by intervening in the metabolism of proteins and nucleic acids, in the enzymatic processes, besides ensuring a good functioning of the immune system, contributing to the healing of wounds.	(Delgadinho, 2014)
	This metal also has the antioxidant function, being part of the structure of the antioxidant enzyme superoxide dismutase (SOD), present in the cytoplasm of all cells. Thus, it protects all cells exposed to oxygen against oxidative damage caused by free radicals (by the superoxide) through their neutralization, that is, the catalysis of superoxide radical dismutation occurs.	(Azziz, 2011)
	In its ionic form, zinc plays a key role in synaptic	

	function and neurotransmission.	
Selenium	Selenium is a mineral antioxidant that prevents oxidative damage induced by free radicals, working together with vitamin E. Selenium plays an important role in the immune system since it is closely associated with the concentration of the GPx enzyme. Thus, hight GPx concentrations result in at least one predisposition for tumor development. In addition, this mineral is very important for the male reproductive system.	(Food Ingredients Brasil, 2009; Carvalho, 2005).

II. 6. Antioxidant properties of mushrooms

Investigators have been searching for new natural and non-toxic products with antioxidant activity. Some biomolecules capable of stimulating or suppressing the immune system, as well as nutritional and medicinal properties have been found in many mushrooms. Mushrooms cover a wide diversity of fungi and are known for its functional attributes such as for the degradation of organic matter, the perfect symbiosis they establish with the roots of plants (Lauw *et al.*, 2013), but may also be pathogenic for many trees, causing root rot diseases (Coetzee *et al*., 2015).

Mushrooms are a good food choice as they are very high in fiber and protein content. They are composed essentially of water (80 - 90 %), are low in fat and carbohydrates, as well as low cholesterol and low calories. They are therefore considered to be one of the most "complete" foods, presenting a nutritional value being similar to that presented by milk and by meat and to be significantly more nutritious than most vegetables (Smina *et al.*, 2011; Robaszkiewicz *et al*., 2010; Carvalho, 2005).

As previously mentioned, mushrooms are a natural source of a wide variety of antioxidants, which enable them to neutralize free radicals, some of them unique to a particular mushroom. Antioxidant compounds can be found in fruiting body, mycelium, and spores.

In following sub-chapters, will be made a presentation of the mushrooms to be studied. *Ganoderma lucidum* and *Ganoderma lingzhi* were selected, due to its worldwide use as medicinal mushroom and because its significant antioxidant activity that have. *Boletus*

edulis was chosen because it is a very consumed species and it is interesting to widely study its medicinal properties, because can be used in the prevention of cancer. *Tricholoma equestre* was also chosen, although it is not advised to consume, because possess compounds of interest that can be applied in other areas, such as supplements or medicines.

II. 6.1. *Ganoderma lucidum* and *Ganoderma lingzhi*

The mushrooms known as "Lingzhi" in Chinese or "Reishi" in Japonese are mushrooms used for thousands of years in traditional medicine in China and other Asian countries due to their medicinal properties (Hennicke *et al*, 2016).This mushrooms it is commonly called "the mushroom of eternal youth" or "the mushroom of immortality" if consumed regularly (Russell, 2006; Sanodiya *et al*., 2009) and symbolizes success, longevity, goodness and sanctity (Cao *et al.*, 2012).

These mushrooms contemplates a numerous pharmacological effects, namely immunomodulatory action, anti-inflammatory effect, antitumor activity, chemo preventive, anti-diabetic (Sanodiya, *et al.*, 2009). Other studies have demonstrated has a therapeutic properties, such as antioxidative and radical scavenging, antiaging effects, besides increasing memory and enhancing vital energy (Wachtel-Galor *et al.*, 2011). Besides that, because of their anti-viral, anti-bacterian and anti-fungic activities, they are referred to be used in treatment of viral infections, especially HIV (Sanodiya *et al.*, 2009; Boh *et al.*, 2007). In addition, there are pharmacological studies that have revealed their activity on the central nervous system (Zhu *et al.*, 1999). They also have been used for the prevention or treatment of a numerous diseases including coronary heart diseases, hypercholesterolemia, hepatitis, hypertension, asthma, allergies (Boh *et al.*, 2007; Wachtel-Galor *et al.*, 2011; Paterson, 2006).

Smina *et al*. (2011) reported various types of antioxidants from the *Ganoderma* which can reduce oxidative damage by directly scavenging free radicals generated in the cell. They contains a complex set of hundreds of biologically active molecules, that including triterpenes, polysaccharides, nucleotides (adenosine), steroids, fatty acids, proteins, peptides, amino acids and minerals (like as calcium, copper, germanium, iron, magnesium, manganese, potassium, selenium, zinc). This mushroom in addition to the compounds already mentioned, contains also complex B vitamins, phenols and others components present in the fruiting body, mycelium, and spores that together confer innumerous benefits which promotes human health and life quality (Huie and Di, 2004; Cukalovic *et al*., 2010; Wachtel-Galor *et al*., 2011). The complex B vitamins are very important for the health of the nervous system. The

regular consumption helps protect and improve memory, as well as avoid problems of cognitive decline, slow thinking, and dementia (The Reader's Digest Association, Inc., 2017).

Analysis carried out in mushrooms also indicated the presence of vitamins C and E, as well as beta-carotene, which reinforces the idea that these mushrooms are true sources of antioxidants (Paul *et al*., 2014; Stojković *et al*., 2013; Acharya *et al*., 2015 Yegenoglu *et al*., 2011).

Ganoderma lucidum, the generic type, although originally reported from the UK, presents in the present, a worldwide distribution, appearing in Europe, Asia, America, Oceania and Africa (Moncalvo *et al.*, 1995; Cao *et al.*, 2012). Nowadays, thanks to advances in cultivation techniques, these mushrooms can be cultivated artificially, using, for example, substrates such as grains, sawdust, wood logs and cork residues(Wachtel-Galor *et al*., 2011). In this way, the mushroom *Ganoderma lucidum* it becomes more available and can even be marketed, also in the form of extracts (Boh *et al.*, 2007; Wachtel-Galor *et al.*, 2011).

The name *Ganoderma lucidum* was erroneously applied to other similar *Ganoderma*, including the Lingzhi fungus in East Asia. During many years, the designation of the famous medicinal fungus known as "Lingzhi" in East Asia was exclusively attributed to *Ganoderma lucidum*, a species originally described in Europe (Moncalvo *et al.*, 1995). However, it were performed molecular studies based in nuclear RNA, in which were performed detailed morphological and phylogenetic study of various *Ganoderma* species that mostly resemble "Lingzhi". These analysis clearly indicated that *Ganoderma lucidum* from East Asia represents a different species from *Ganoderma lingzhi*, that is, they are distinct mushrooms. Thus, a new denomination was established for the mushroom of Chinese origin, being named *Ganoderma lingzhi,* while *Ganoderma lucidum*, was entrusted to the mushrooms coming from Europe and North America (Cao *et al.*, 2012).

In addition, the external and morphological characteristics presented are very distinct which also points in the direction of diversity. The *Ganoderma lingzhi* has yellow pore surface and have a taste bitter, while the *Ganoderma lucidum* has a white pore surface and woody making so very difficult to be edible can being consumed in the form of teas or in some cases combined with other foods or natural products (Sanodiya *et al*., 2009). In other study performed in ethanol extract of ground basidiocarps showed that have a content of triterpenic acid different, with the *Ganoderma lingzhi* presenting a greater content of this molecules (Hennicke *et al*., 2016). The same study, showed a higher diversity and higher amounts of ganoderic acid in *Ganoderma lingzhi* than in *Ganoderma lucidum* that probably are responsible for the bitter taste. Besides that, the triterpenoid contents is rather low in

Ganoderma lucidum, which makes it very likely that this mushroom has no medicinal properties, despite also contain other bioactive compounds like polysaccharides.

Figure 7 show the external differences of each species of *Ganoderma*.

Figure 7 - Fruiting bodies (dryed) of *Ganoderma lucidum (left)* and *Ganoderma lingzhi (right).*

It should be noted that there is not yet much information available on the species *Ganoderma lingzhi*.

II. 6.2. *Tricholoma equestre*

As already mentioned, mushrooms are nutritionally very rich foods, in addition to allowing beneficial health effects in general, since they contain molecules with some antioxidant power in their structure.

Tricholoma equestre is a mushroom very typical of center of Portugal is the yellow "míscaro" which is considered one of the *ex-libris* products in the gastronomy and local economy, being able to be found in fairs and markets of the region.This mushroom is among the species most appreciated and most sought by the lovers of the regional gastronomy, in Portugal, being the main ingredient of the traditional rice of "míscaros" (Henriques, 2008).

It is commonly known by different names, namely, "míscaro" or "tortulho", exhibiting, when seen from above, a canary yellow cap, sometimes with a brownish touch, with a diameter between 5 and 12 cm. It is easily distinguished from other species by having yellow slides and feet, generally of a length of between 4 and 10 cm and a regular diameter. It is further emphasized that it is coated by a thick and viscous, but separable, cuticle that must be removed, when it is intended to consume (ICNF, 2013).

Figure 8 - Fruiting body of *Tricholoma equestre*, in the field. (Taken from https://pt.wikipedia.org/wiki/Tricholoma_ equestre. Accessed March 2017).

The "míscaro", whose scientific name is *Tricholoma equestre*, also known by *Tricholoma flavovirens*, is a wild mushroom that is famous for preferring certain plant species, appearing with some frequency in the *Pinus Pinaster* pine forests, mainly in sandy lands. This species can be easily found, since it is known the optimal conditions for its growth, and can be harvested after the first rains, during autumn and winter.

Although it is a mushroom appreciated by many, *Tricholoma equestre*, began to be consumed in lesser quantity and frequency, and has even been banned in many European countries because it is suspected that it is a very toxic species (ICNF, 2013; Ribeiro *et al*., 2006). Several cases of intoxication have been reported, some of them fatal, in France (12 cases) and Poland (2 cases), caused by the ingestion of large doses in consecutive meals (Bedry *et al.*, 2001, Chodorowski *et al*., 2002). This mushroom contains a toxin called cycloprop-2-ene carboxylic acid (Figure 9), which accumulates in the body when significant amounts of this mushroom are ingested and which is responsible for degenerative muscle disease, rhabdomyolysis (Matssura *et al*., 2009.)

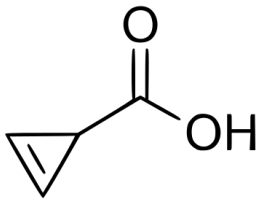


Figure 9 - The molecular structure of the cycloprop-2-ene carboxylic acid.

Tricholoma equestre is presently considered a toxic mushroom, and its consumption is not advised, although a normal and moderate consumption of this, in small quantities, seems to have no obvious implications for human health (Boucard, 2001). However, since it contains some compounds of interest, these can be extracted and used in other applications, namely in cosmetics.

II. 6.3. *Boletus edulis*

The mushrooms *Boletus edulis* are among the safest edible wild species and most often harvested in Central European countries, including in Portugal (Jaworska and Bernas, 2009).Its popularity is mainly due to its sensorial qualities, such as aroma, flavor and texture, being considered one of the best edible species and the most delicious of all the species belonging to the genus *Boletus* (Jaworska and Bernas, 2009).These mushrooms can to be are prepared in the most diverse forms, leading to dishes with a delicious and tasty appearance (Zhang *et al.*, 2015).

This mushroom, besides being delicious, has a low content of fat and digestible carbohydrates and is very nutritious due to its richness in proteins, vitamins, minerals, amino acids, polysaccharides and dietary fiber (Vamanu and Nita, 2013; Luo *et al*., 2012).

In Portugal, these mushrooms are known by popular such as highlight "boleto", "cabeçudo", among others (Fernandes,1990). It is a very frequent and abundant species in Portugal, particularly in the highlands, when the environmental conditions are favorable, being one of the species most commonly consumed in the regions of Minho, Douro, Beiras, Trás-os-Montes and Alentejo, as well as *Tricholoma equestre* (Carvalho, 2005).

The *Boletus edulis* mushrooms may appear individually or in small clusters of two or three mushrooms, in the late summer early autumn, not being tied to a particular habitat, and may be found near coniferous roots (pines and firs), oak, chestnut or cork oak, in generally acidic soils (ICNF, 2013; Ugon and Manavella, (n.d.); Garnweidner, n.d).

The *Boletus edulis* present a great variability and a wide distribution, existing numerous species, subspecies and forms very close to *Boletus edulis*, however, can hardly be confused with any other from the same family as long as the color of his cap and tubes, at first white, are observed meticulously, turning yellow and green (Muñoz, 2005; Garnweidner, n.d). Nevertheless, this mushroom is also a confusing species in Nature with other mushrooms like as *Boletus aereus*, *Boletus reticulatus*, *Boletus pinophilus* and/or *Tylopilus felleus* (ICNF, 2013; Garnweidner, n.d).

Boletus edulis has a convex fleshy hat with a diameter that can vary between 10 and 25 cm in shades ranging from light brown to dark brown, sometimes with brownish-reddish (Figure 10). However, in very young specimens, hats may have an almost whitish hue. As for the meat, this is white and is directly under a cuticle also brownish and has a great consistency in young mushrooms. The size of their stipe vary between 5 to 15 cm in length and are whitish or light brown in color (Cardoso, 2006).

Figure 10 - Fruiting bodies of *Boletus edulis* in the field.

Like other edible mushrooms, *Boletus edulis* are very perishable, due to their high water content, the high level of enzymatic activity and also due to the presence of microflora, and some care must be taken in their handling and storage (Jaworska and Bernas, 2009).

Studies have revealed that fruiting bodies of *Boletus edulis* have high antioxidant power due to the combination of different organic acids such as oxalic, citric, fumaric, succinic and malic acid, ergosterol and ergothionein (Muszynska*et al.*, 2013). Other studies performed with extracts of this fruiting bodies mushroom showed a strong free radical scavenging activity (Sarikurkcu *et al.*, 2008). Pharmacological studies have demonstrated that the polysaccharides extracted from fruiting bodies of these mushrooms have antitumor effect on renal cancer in mice and years later, it was proven that prevents the proliferation of human colon cancer cells (Wang *et al.*, 2014). Additionally, yours polysaccharides are responsible by possess antioxidant and anti-inflammatory properties (Wang*et al.*, 2014; Lemieszek *et al.*, 2013).

II. 7. Active substances in mushrooms

The medicinal and antioxidant properties generally attributed to mushrooms are due to the presence of a variety of bioactive substances, such as polysaccharides, of which the most important are β-glycans, which are attributed the main antioxidant effects of mushrooms (Kozarski *et al*., 2015). However, the presence of phenolic compounds also may have direct contribution in antioxidant action of mushrooms, due to the scavenging ability provided by the hydroxyl groups (Kim *et al*., 2008). Sources indicated that poliphenols were considered as the major naturally occurring antioxidant compounds in the wild edible mushrooms (Wang and Xu, 2014). Table 2 shows the different antioxidant compounds that can be found in the studied mushrooms of which stand out phenolic compounds and polysaccharides.

Table 2 - Antioxidants compounds present in mushrooms *Tricholoma equestre, Boletus edulis* and *Ganoderma lucidum.*

Mushroom scientific name	Antioxidant compounds	Biomaterial source	References
Tricholoma equestre	Phenolic compouds (*p*-hydroxybenzoic acid) Beta-carotene	Aqueous and methanolic extracts of dried fruiting bodies	(Ribeiro *et al*., 2006; Robaszkiewicz, 2010)
Boletus edulis	Beta-carotene Ascorbic acid Flavonoids Tocopherols	Fruiting bodies extracts	(Ribeiro *et al*., 2006)
	Phenolic compounds (p-hydroxybenzoic acid, protocatechuic acid,gallic acid, cinnamic acid, p-coumaric acid, caffeic acid)	Aqueous extract	(Wang and Xu, 2014; Muszynska *et al.*, 2013; Alves *et al*., 2013)
Ganoderma lucidum	Triterpenoids Polysaccharides Phenolic compouds (p-hydroxybenzoic acid, protocatechuic acid,gallic acid, cinnamic acid, p-coumaric acid, caffeic acid) Flavonoids	Fruiting bodies Mycelium	(Kozarski *et al.*, 2011; Alves *et al*., 2013)

II. 8. Extraction processes of mushrooms compounds

Extraction processes enable the separation/ isolation and concentration of groups of compounds of interest that are present in plants, fruits and vegetables. Appropriate extraction methods have been developed and optimized according to the extractable ingredients, in order to obtain higher extraction efficiency of specific compounds.

Many factors interfere in operational conditions and solvent selection is a relevant aspect, since stability of the extracted substances may be affected. Furthermore, issues such as selectivity, toxicity, availability, cost and handling risks must be also taken into account.

A variety of solvents are available (e. g. methanol, ethanol, acetone, water (hot or cold), chloroform, petroleum ether, *n*-hexane and toluene), being that solvent/solvent and solvent/solute ratios are defined according to the extractable compounds and solvent chosen. There are also other factors that affect the efficiency of the extraction process, such as agitation, temperature, duration, number of extractions and techniques used during the extraction (filtration, sonification).

In Table 3, a bibliographical review is made about the conditions that are necessary to extract the compounds of interest present in mushrooms.

Table 3 - Necessary conditions for the extraction process of the antioxidant compounds of interest.

Compound extracted **Mushroom**	**Conditions of the extraction process**	**References**
Polisacharides ***Ganoderma lucidum***	Lyophilized mushrooms (~ 1.5 g) Extraction with water (50 mL) at boiling temperature for 2h and with stirring (150 rpm). After filtration, the residue was then extracted with two more portions of boiling water. Combined extracts were lyophilized, then 95 % ethanol (10 mL) was added and polysaccharides were precipitated overnight at 4 °C. Precipitated polysaccharides were collected after centrifugation at 3100 ×g for 40 min followed by filtration, and finally, were lyophilized, resulting in a crude polysaccharidic sample.	(Heleno *et al.*, 2012)
Phenolic compounds ***Ganoderma lucidum***	Lyophilized mushrooms (~ 1 g) Extraction with 80 % methanol (30 mL) at -20 ºC for 2h. Followed by sonification (15 min) and filtration. The residue was then extracted two more times with 30 mL of 80 % methanol.	(Heleno *et al.*, 2012)

	The resulting extracts were combined and evaporated at 40 °C under reduced pressure (rotary evaporator) to remove methanol. Afterwards, the aqueous phase was washed with *n*-hexane, and then subjected to a repeated liquid-liquid extraction with diethyl ether (3×30 mL) and ethyl acetate (3×30 mL). Organic phases were evaporated at 40 °C to dryness and re-dissolved in a solution of 80 % methanol.	
Phenolics and Organic Acids ***Tricholoma equestre*** ***Boletus edulis***	Powdered mushroom (10 g) Extract with water (500 mL) at boiling temperature during 30 min filtered afterwards (Büchner funnel) The resulting extracts were lyophilized and kept in an desiccator, in the dark, at room temperature.	(Ribeiro, B. *et al*., 2006)
Polysaccharides ***Boletus edulis***	Powdered mushroom (100 g) Extract with petroleum ether for 2 h, and further extracted with 80 % ethanol at 90 °C for 2 h. After filtration, the resulting filter cake was extracted three times with double distilled water at 100 °C for 2 h. The extracts were combined, concentrated using a rotary evaporator at 55 °C and filtered. Afterwards, the resulting extract was deproteinized three times applying the Sevag reagent. The absence of proteins in the polysaccharide was evaluated through the signal of the UV spectrum at 280 nm. Then the reagent was removed and the extract precipitated by adding ethanol (four times the volume of the aqueous extract), being the mixture kept overnight at 4 °C to yield the polysaccharide. The precipitate was collected	(Luo *et al*., 2012)

	by centrifugation at 4000 rpm for 10 min, washed successively with petroleum ether, acetone and ethanol, and the precipitation procedure was repeated. Then the precipitate was dissolved in water, was dialyzed against deionized water for 72 h and was freeze-dryed to yield the crude polysaccharide.	
Polysaccharides ***Boletus edulis***	Powdered mushroom (100 g) Extract with 80 % ethanol (700 mL) at 80 ºC (boiling temperature) during 1h with continuous stirring. After this, the insoluble material was filtered using glass fiber and washed twice with 50 mL of 80 % ethanol. AIR (Alcohol Insoluble Residue) was dried in a forced air oven at 50 ºC for 12 h. 100 g of AIR were added to 700 mL of boiling water, being the suspension boiled with stirring for 1 h. Next, the insoluble material was separated from the supernatant by centrifugation (6000 rpm, 20 min, 4 ºC), washed with water and separated again by centrifugation, as before, two more times. The supernatants were combined and concentrated in a rotary evaporator at 35 ºC. Finally, a dialysis step was performed and the resulting solution was lyophilized.	(Lemieszek, M.K. *et al.*, 2013)

III. Materials and methods

III. 1. Origin of the study mushroom samples

All mushrooms studied were from Portugal. The cultivated mushrooms *Ganoderma lucidum* and *Ganoderma lingzhi* were provided, dried, by the company Quadrante Natural, Lisbon. The wild mushrooms *Boletus edulis* were obtained from Professor Guilhermina Marques, from the Department of Agronomy, UTAD, Vila Real. They were collected in November 2016, in a mixed stand located in Alfândega da Fé, Bragança, and maintained frozen until the analysis. The *Tricholoma equestre* mushrooms were collected last winter in a forest near Cantanhede, and frozen after being cleaned and cut in fragments. The mushrooms were then freeze-dried (Dura-Dry TM MP Microprocessor Control Corrosion Resistant Freeze-Dryer) in order to remove the water, milled to a fine powder that was stored in a cool and dry place.

III. 2. Reagents

The extraction of phenolic compounds and polysaccharides was carried out using ethanol (96 %, AGA - Álcool e Géneros Alimentares, SA, Prior Velho, Portugal).

The total phenolic content was determinedusingFolin-Ciocalteu reagent (Panreac Química SLU, Barcelona, Spain), sodium carbonate, Na_2CO_3 (Merck) and gallic acid, $C_7H_6O_5$ (Panreac Química SA, Barcelona, Spain).

The HPLC technique was performed using methanol (≥ 99,9 %, Sigma-Aldrich).

For determination of polysaccharides content was used 2-Deoxy-D-glucose, $C_6H_{12}O_5$ (≥ 98 %, Sigma Aldrich), D-(+)-Mannose, $C_6H_{12}O_6$ (≥ 99 %, Sigma Aldrich), L-Rhamnose monohydrate, $C_6H_{12}O_5.H_2O$ (≥ 99 %, Sigma Aldrich), D-(+)-Glucose anhydrous for biochemical purposes,$C_6H_{12}O_6$ (Merck), D-Galacturonic acid sodium salt, $C_6H_9NaO_7$ (≥ 98 %, Sigma Aldrich), D-Glucuronic acid, $C_6H_{10}O_7$ (≥ 98 %, Sigma Aldrich), D(+)-Xylose, $C_6H_{10}O_5$ (≥ 99 %, Merck), D-(-)-Arabinose, $C_5H_{10}O_5$ (≥ 98 %, Sigma Aldrich), D-(+)-Galactose, $C_6H_{12}O_6$ (≥ 99 %, Sigma Aldrich), D(-)-Rybose, $C_5H_{10}O_5$ (Merck).

All previous solutions were prepared using distilled water (New Water Purification System) with an electrical conductivity of about 12.7 μS.cm.

The ACSF solution was prepared using sodium chloride, NaCl (99.7 %,José Manuel Gomes dos Santos, Lda, Odivelas), sodium bicarbonate, $NaHCO_3$ (≥ 99.7 %, Sigma-Aldrich, Sintra, Portugal), D-(+)-Glucose (≥ 99,5 %, Sigma-Aldrich, Sintra, Portugal), potassium

chloride, KCl (≥ 99.0 %, Sigma-Aldric, Sintra, Portugal), magnesium chloride hexahydrate, $MgCl_2.6H_2O$ (Merck, Lisbon, Portugal), sodium phosphate monobasic dihydrate, $NaH_2PO_4.2H_2O$ (≥ 99 %, Sigma Aldrich, Sintra, Portugal), calcium chloride dihydrate, $CaCl_2.2H_2O$ (≥ 99 %, Sigma Aldrich, Sintra, Portugal). The ACSF medium was preparede with ultrapure water (INTERLAB, Lisbon, Portugal) with a resistivity of 18.2 MΩ.cm.

III. 3. Extraction procedure

The extraction of the compounds of interest, phenolic compounds and polysaccharides, was made using the methods (briefly) described next.

Phenolic compound extract

To the 0.5 g sample of the lyophilized and powdered mushrooms material was added 50 mL of a mixture of ethanol and water (4:1). This mixture was agitated for 1 h, and centrifugated at 7000 rpm during 10 min and the resulting supernatant was collected. The previously described procedure was repeated twice. Next, the supernatants were filtered and filtrate was concentrated through a rotary evaporator to remove the entire ethanol. The concentrated extract was solved in 100 mL of distilled water and frozen. After, the extracts were freeze-dried and kept in a dark and cool place until further analysis.

Polysaccharide extract

To 350 mL of a solution with 80 % ethanol at 80 °C, was added 50 g of the lyophilized and powdered mushroom sample being the resulting suspension boiled for 1 h with stirring. The insoluble material was filtered under vacuum using a filter paper and washed several times with 100 mL of the 80 % ethanol solution, until clear liquid was obtained. AIR (Alcohol Insoluble Residue) was dried in a forced air oven at 50 ºC until totally dry, ensuring that ethanol was removed in its entirety. After this, to 350 mL of boiling water, was added 50 g of AIR, and the suspension was boiled with stirring for 1 h. The resulting suspension was centrifuged at 6000 rpm, for 20 min, at 4 °C and the supernatant was separated. Water insoluble material was washed with water (4 or 5 times using approximately 30 mL) and separated again by centrifugation as before. The supernatants (WSM - water soluble material) were combined and were subjected to an extra filtration step, using a kitazate and filter paper, in order to obtain a clearer solution for an easier evaporation of the water. The filtrate was

concentrated using the rotary evaporator until a very concentrated solution was obtained. Thereafter, the concentrated solution was dialysed against distilled water (6 water renewals), frozen, freeze-dried yielding the WSB (water soluble biopolymers) and kept in a dark and cool place until further analysis.

III. 4. Total phenolic content (phenol content)

A diluted sample containing 1 mg of lyophilized mushroom extract and 1 mL of distilled water was prepared. To it 0.5 mL of Folin-Ciocalteu reagent, 1 mL of 7.5 % sodium carbonate and 7.5 mL of distilled water were added. The resulting solution was allowed to stand in the dark for 1 h and at the end of that time, the absorbance was measured at 765 nm (Lambda 25 UV / VIS Spectrometer, Perkin Elmer). Gallic acid was used to obtain the standard curve (0-150 mg/L), being the results expressed in mg GAE / g of extract.

III. 5. Phenolic compounds

A solution composed by 10 mg of phenolic compounds extract and 1 mL of a mixture of 50 % methanol was used to obtain chromatographic profiles. The chromatographic separation was performed using a system of High Performance Liquid Chromatography (Ultimate 3000, Dionex), operating at 35 ºC during 75 min, with a flow rate of 1 mL / min, and an injection volume of 50 μL / min. The mobile phase used was a mixture of formic acid 5 % (phase A) and methanol (phase B) and the composition gradient was 5 % methanol until 65 min being changed to 65 % and 5 % methanol at 65 and 67 min, respectively. The mobile phase was monitored at 280 nm using a photodiode array detector PDA-100, Dionex.

III. 6. Monosaccharide content

For the analysis of the monosaccharide content, through ion exchange chromatography, it was necessary to proceed with the hydrolysis of the polysaccharide material, adding sulfuric acid. For this, a solution containing 5 mg of mushroom polysaccharide extract and 200 μL of a 72 % solution of sulfuric acid, was previously prepared. Then, 3 glass spheres were placed inside the tubes and allowed to stand at room temperature for 3 h, samples mixed in vortex (IKA® Vortex 3) each 15 min. After, 2 mL of ultrapure water were added being the mixture placed in a heating block (Sample Concentrator, Techne®) for 2 h 30 min and it was cooled overnight. After, to 0.5 mL standard solution,

composed by 1 mg of 2-deoxy-D-glucose and 1 mL ultrapure water, were added to solution that was cooled during night and homogenised.

Chromatographic separations and sugars analysis were performed using a Dionex Instrument ICS-3000 Ion Chromatography System at 35 ºC using an eluent flow of 0.30 mL / min for 52 min. The composition gradient was 7.5 % of eluent B (10 mM NaOH, 2mM $Ba(OH)_2$) until 19 min and was changed to 7.5 % of B and 50 % of eluent C (1 M acetate, 2mM $Ba(OH)_2$) and 40 % of eluent D (500 mM NaOH, 2 mM $Ba(OH)_2$) at 27 and 43 min, respectively.

For the quantification of the sugars Arabinose, Fucose, Galactose, Glucose, Mannose, Rhamnose, Xylose and Ribose, solutions with these sugars were prepared with a concentration of 10 mg / mL and from these, standard solutions were made using different volumes that varied between 25 and 250 μL for sugar standards and 500 μL for internal standard (1 mg / mL of 2-deoxy-D-glucose). The quantification of the sugars in the samples was obtained by the internal standard method.

III. 7. Presence of RNA

In order to verify the presence of RNA in the polysaccharide extract of the studied mushrooms, a spectral scanning in the UV / VIS region was performed. For this purpose, a solution composed of 1 mg of mushroom polysaccharide extract and 1 mL of distilled water was prepared. Afterwards, 100 μL of this solution was added to 1 mL of distilled water. The resulting solution was analysed in a spectrophotometer (Lambda 25 UV / VIS Spectrometer, Perkin Elmer) between the wavelengths 200 and 700 nm.

III. 8. Qualitative elementary analysis / Elemental analysis (X-ray fluorescence spectrometry)

For a better chemical characterization of the *Boletus edulis*, *Tricholoma equestre*, *Ganoderma lucidum* and *Ganoderma lingzhi* mushrooms, the elemental composition of mushroom samples was evaluated using the X-ray fluorescence spectrometry technique (XRF). For elemental determination was used a High Sensitivity Fluorescent X-ray analyzer SEA6000VX, HSFinder, Hitachi.

XRF analysis is sensitive to elements with atomic number superior to that of sodium ($Z > 11$), belonging to the periodic table. In addition, it allows to explore and analyse the

different metallic elements present in the mushrooms, in a non destructive way, in which the matrix is analyzed without its disintegration.

For analysis, the mushrooms samples were placed, pressed and compacted in a sample holder and, with the aid of a glass slide, a smooth, flat and homogeneous surface was created.

Each sample holder was exposed directly to the X-ray beam and the analysis proceeds for 60 seconds being that the area of the sample where the X-ray focus is 3× 3mm. The X-ray are detected through a multi-cathode Si semiconductor detector with a resolution until 185 eV.

Qualitative analyzes were made, using two different energies (15 and 50 keV) in order to identify the maximum number of chemical elements present in the mushrooms. The lower energy (15 keV) identifies lighter chemical elements while heavier elements are identified when the analysis is performed using higher energy (50 keV).

III. 9. Experimental arrangement and optical measurements

To acquire optical signals, by transfluorescence, was used a fluorescence microscope (Zeiss Axioscop) with a halogen light source (12V, 100W) which is shown in Figure 11.

The light emitted by this lamp passes through an excitation filter, which allows only the passage of light with wavelengths exceeding 480 nm. It then passes through a mirror at an angle of 45° and a set of lenses that will allow to focus the light that will affect and excite the biological preparation that is in the experimental chamber.

In addition to the filters, there are also two diaphragms, the field diaphragm that allows adjusting the illumination of the preparation and the opening diaphragm that allows limiting the region where the data is collected.

The light is focused by immersing a lens with a magnification of 40× in water and a working distance of 1.6 mm. The light emitted by the preparation then passes through a 500 nm high pass filter that allows the light to be selected in the region of the visible and remove the contamination caused by the incident light, passing only the light with wavelengths above 500 nm.

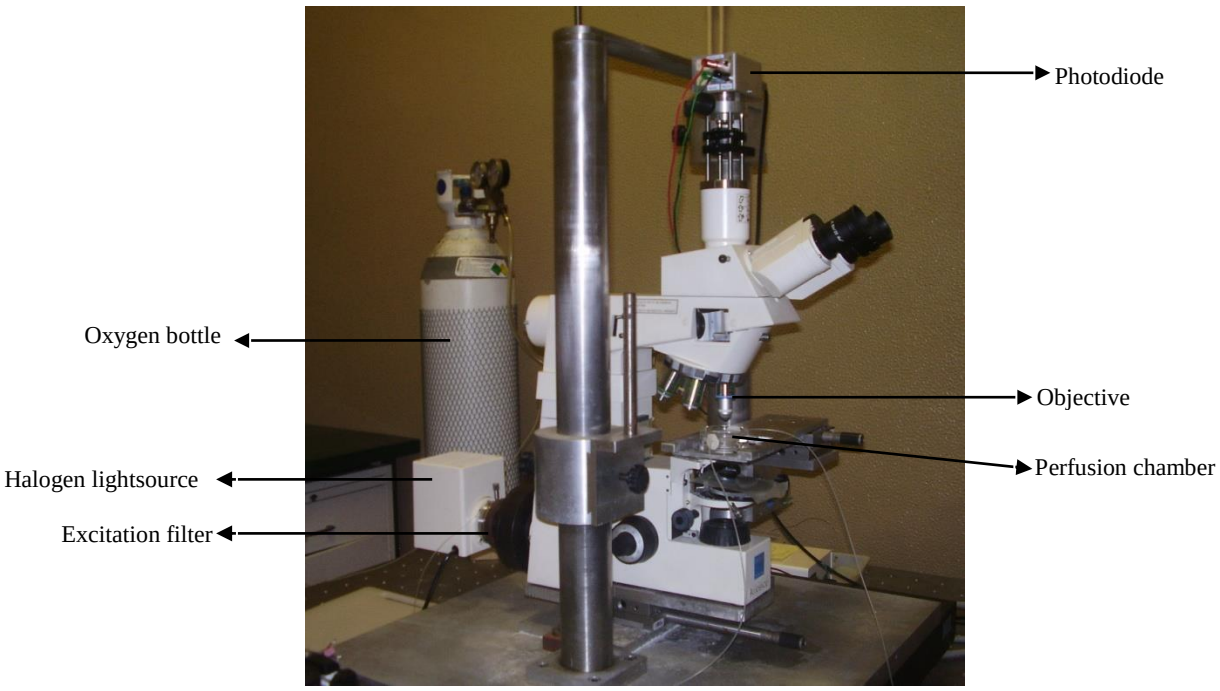


Figure 11 - Principal components of fluorescence microscope and system of the oxygenation.

The resulting fluorescence signals, detected by a silicon photodiode (HAMMAMATSU-S1226-Serie, with an area of 1 mm^2), are then converted into an analog potential voltage signal (measured in mV) through a current to voltage (I/V) converter of 16 bits. Once collected and converted, the signals are processed in a computer by a data acquisition system (Figure 12) with the help of the software National Instruments Signal Express 2013.

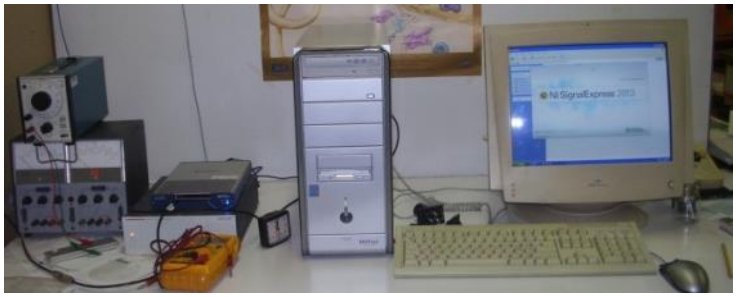


Figure 12 - Data acquisition system.

For perfusion of the liquid in the experimental chamber a peristaltic pump (GILSON-MINIPLUS-3, Middleton, USA) was used, which allowed the circulation of the extracellular medium at a constant rate of about 1.5 a 2 mL / min, at a constant temperature of 37ºC (Figure 13).

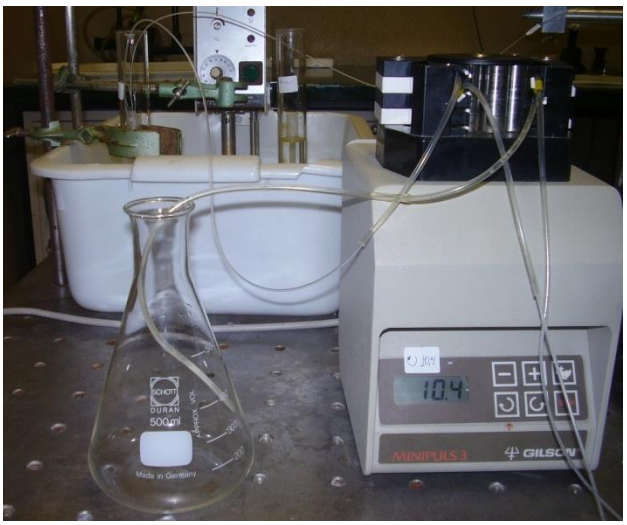


Figure 13 - Peristaltic pump and the bath maintained at a constant temperature of 37°C.

III. 10. Dissecting and obtaining hippocampal slices

In this study, are used females of Wistar rats, aged between 8 and 16 weeks, and in some cases, they are pregnant for 16-18 days.

Animals are sacrificed by cervical dislocation, followed by decapitation. Then, with the aid of scissors, the skin of the head and the bones of the skull are removed from the occipital to the forehead along the sutures of the parietal bones. After, with the aid of a spatula, and through a surrounding movement, the brain is separated from the cranial nerves and the optic nerves.

The brain, presents in Figure 14, once isolated, is placed immediately in an ice-cold solution (5 - 8 ºC) of artificial cerebrospinal fluid (ACSF), previously oxygenated (95 % O_2 and 5 % CO_2).

Subsequently, the brain is placed on an icy Petri dish on a filter paper previously bathed with ACSF to proceed to the separation of the two cerebral hemispheres.

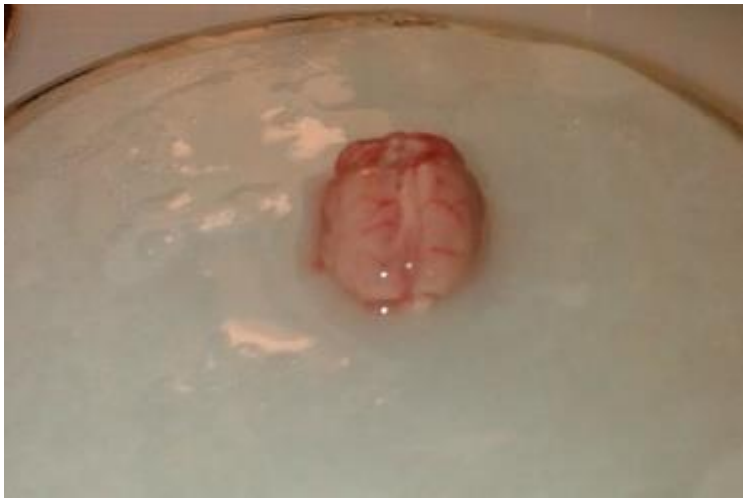


Figure 14 - Wistar Rat Brain.

During the procedure, as described below, frozen solution of chilled ACSF is placed on the biological preparation, in order to prevent cell death. You start by put the brain on an icy Petri dish on a filter paper previously bathed with ACSF to proceed to the separation of the two cerebral hemispheres. After, with the help of a scalpel, was made a cut between the two hemispheres, thus dissecting the hippocampus. Then is also made a cut on the fimbria and another on the entorhinal cortex, and with the aid of a spatula the hippocampus is carefully separated from the remaining tissues.

Subsequently, the hippocampus is placed on the same Petri dish and, in order to allow easier access to the hippocampal CA3 and CA1 regions, transverse slices are obtained in the mid-hippocampus zone, each with a thickness of 400-500 μm, using an instrument with 5 blades and 6 spacers arranged in parallel (Figure 15).

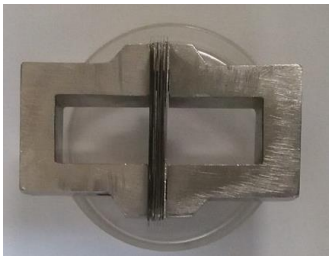


Figure 15 - Instrument used to obtain the slices of the middle zone of the hippocampus.

After, with the help of a fine brush and a spatula, the slices are carefully removed from the slides and, in order to ensure their survival for several hours, they are placed in an incubator vessel with oxygenated ACSF solution (95 % O_2 and 5 % CO_2) at room temperature.

This procedure is repeated for the other hemisphere as well.

III. 11. Obtaining ROS optical signals

For evaluating interaction of phenolic compounds and polysaccharides with reactive oxygen species, it was used slices incubated and oxygenated for one hour in a solution of ACSF contained H2DCFDA indicator. The H2DCFDA solution (20 μM) was prepared by dissolving 9.74 mg H2DCFDA in 2 mL DMSO and then 2 μL of this solution was added to 20 mL ACSF solution. Posteriorly, slices were placed in ACSF solution until to be transferred to the experimental chamber. Here, slices were adjusted so that the beam of light is incident on the CA3 region of the hippocampus, in which are found the synapses of the mossy fibers, as can be seen in the representation of Figure 16.

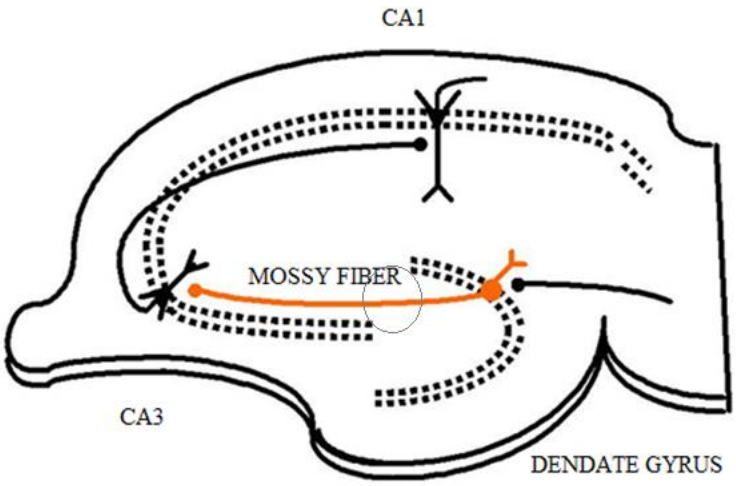


Figure 16 - Representation of a hippocampus slice with indication of the areas of interest. The zone bounded by the curved line indicates the region where the beam is placed (Adapted taken from Purves *et al*., 2004).

ROS signals were observed in the presence of solutions that were prepared containing different concentrations of phenolic compounds and polysaccharides having been collected at the synapses of the mossy fibers of the hippocampus CA3 area.

Experiments begin with the detection of optical signals in slices bathed in ACSF for 30 min. At the end of this period, the slice is perfused by a solution containing the compounds of interest for a period of 30 minutes, and finally, slice are again bathed in the ACSF solution for another 30 minutes. The data were collected with a sampling frequency of 1.66 Hz, corresponding to 100 points per minute, that were average and plotted at 1 min intervals after correcting for the autofluorescence. Thus, according to previous studies, the average of the first 10 minutes of experiment, that form the baseline, are multiplied by a constant equal to 0.7. This resulting value are subtracted in each of the average values per minute and after, divided for the average of the first 10 minutes.

The values resulting from these operations described above are presented by points that corresponding to the normalized mean value of the results obtained in the various experiments ± the standard deviation.

III. 12. Artificial cerebrospinal fluid (ACSF)

Artificial cerebrospinal fluid (ACSF) is an artificial solution that reproduce / simulate the extracellular environment consists of a set of solutions that are prepared in advance, called stock solutions (Figure 17), that are conserved in the refrigerator.

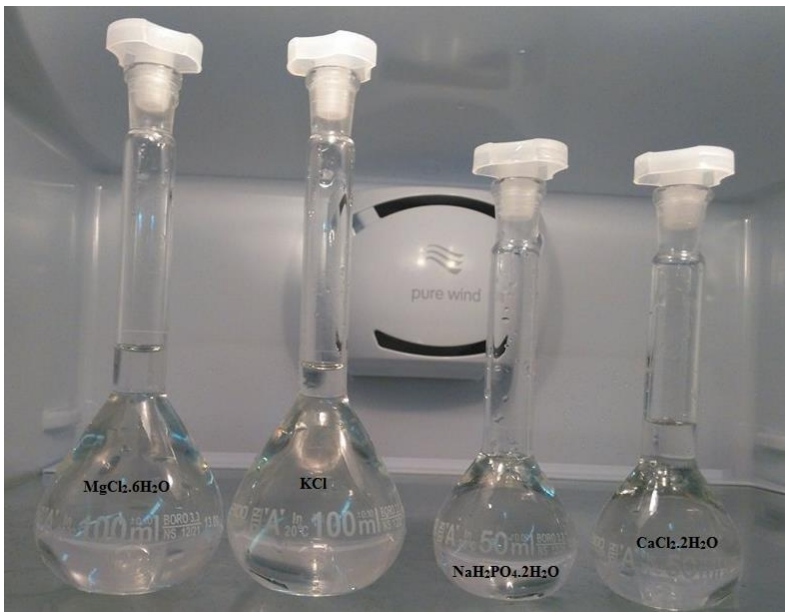


Figure 17 - Stock solutions necessary to prepare extracellular medium.

This solution is prepared in dilution flask with ultrapure water and add the solute compounds as sodium chloride, potassium chloride, sodium hydrogen carbonate, D-glucose, magnesium chloride hexahydrate, sodium hydrogen phosphate dihydrate for calcium chloride dihydrate, in the concentrations indicated in Table 4.

Table 4 - Composition of the ACSF solution.

Compound name	Concentration mM	Molecular weight g mol^{-1}
sodium chloride	124	58.44
sodium hydrogen carbonate	24	84.01
D- glucose	10	180.16
potassium chloride	3.5	74.55
magnesium chloride hexahydrate	2	203.30
sodium hydrogen phosphate dihydrate	1.25	156.01
calcium chloride dihydrate	2	147.02

It should be noted that after preparation of the solution, the pH value of this solution should be checked with the aid of a pH meter (CRISON- MICROPH 2002), which should be approximately 7.4.

At the end, the solution (95 % O_2, 5 % CO_2) was oxygenated for 10 minutes.

III. 13. Preparation of solution extracts

It was started by heating ACSF solution to the bath temperature in order to facilitate the process of dissolving the extract in the ACSF solution so as to allow a greater penetration of the solution into the zone under study and to avoid obstruction of the tubes. In the case of

the extracts from the mushrooms *Boletus edulis*, these had to be subjected to an additional stage of filtration. The resulting solution it was applied in hippocampal slices for 30 minutes, after submitted to an ACSF solution during 30 minutes.

IV. Results and Discussion

IV.1. Phenolic Extracts

IV. 1.1. Yield of extraction of process of phenolic compounds

Table 5 presents the phenolic compounds extracts weights and the corresponding yields for the different strains of mushrooms studied.

Table 5 - Samples and phenolic compounds extract weights and yield from four mushrooms.

Mushroom	Phenolic compounds (g)	Yield %
BE	0.2120 ± 0.008	42.37 ± 1.49
TE	0.2096 ± 0.002	41.88 ± 0.44
G. luc	0.0427 ± 0.011	8.53 ± 2.17
G. ling	0.0275 ± 0.003	5.49 ± 5.49

BE - *Boletus edulis*; TE - *Tricholoma equestre*; G. luc - *Ganoderma lucidum*; G. ling - *Ganoderma lingzhi*
Extracted from dried mushrooms (0.5 g). Data are expressed as mean ± standard deviation (n=3).

Figure 18 represents a bar graph containing the yields corresponding to the extraction of the mushrooms phenolic compounds, using as solvent ethanol.

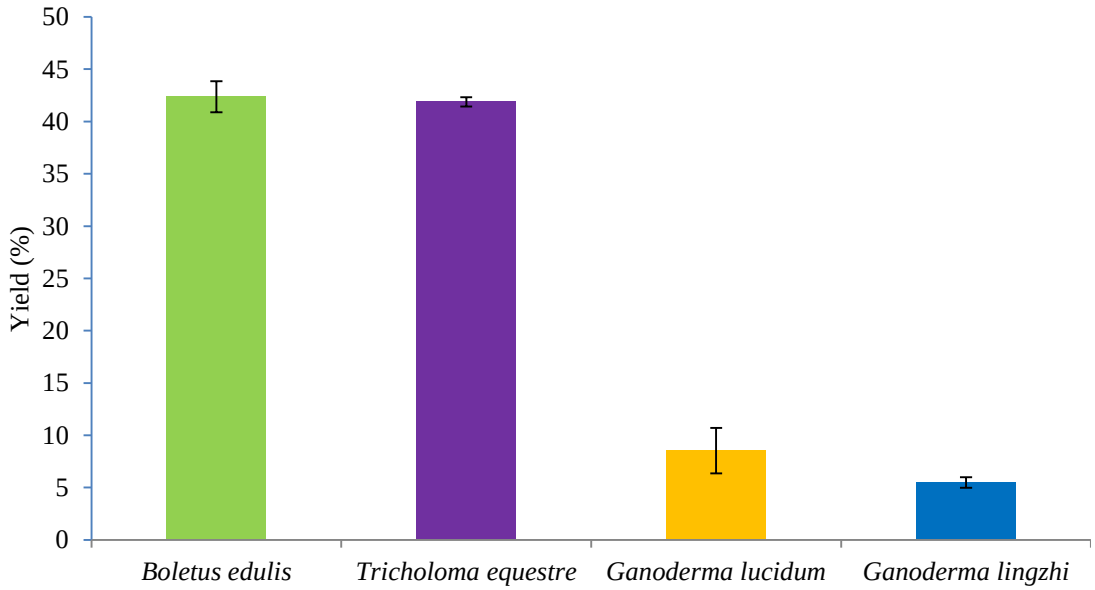


Figure 18 - Yields of extraction process of phenolic compounds for different mushrooms. The solvent used was ethanol**.** Data were expressed as mean ± standard deviation (n=3).

Boletus edulis had the highest phenolics compounds extraction yield, 42.37 %, followed by *Tricholoma equestre* with 41.88 %, while the species *Ganoderma lucidum* and *Ganoderma lingzhi* have lower yields, 8.53 % and 5.49 %, respectively. These values of the extraction efficiencies from the *Ganoderma* species occur in the same range as that obtained

by Ćilerdžić *et al.* (2014), who reported extraction yields between 6.38 % and 8.85 % for etanolic extracts of *Ganoderma lucidum* from different backgrounds. Another work carried out by Mau *et al.*(2002), in methanolic extracts, reported yield values varying from 5.61 % to 6.15 %, that are similar to those found in this study.

Mau *et al.*(2002) have also reported that the yield of the mycelium of the mushroom *Ganoderma lucidum* is close to 40 %, being thus much higher than that of the all mushroom. Furthermore, in another study, (Yang *et al.*,2002) using methanol for the extraction, also obtained yields of 38.1 and 43.9 %, in the white and yellow strains of *Flammulina velutipes*, respectively. They suggest that the high yields obtained, essential due to these mushrooms, in particular, contain higher amounts of soluble sugars and sugars alcohols (Yang *et al.*,2002). The same justification can be used for the similar results obtained in this work for *Tricholoma equestre* and *Boletus edulis*, in which the whole fruiting body of the mushrooms was used.

IV. 1.2. Total phenol content

The quantification of the phenolic content present in each mushroom sample was determined on the basis of a standard curve (Y = 0.023 X - 0.124), where X means the absorbance and Y the concentration in mg GAE.L^{-1}, with a correlation coefficient equal to 0.990. The total phenolic content of the mushrooms samples are shown in Table 6 and Figure 19.

In Table 6, the fourth and fifth columns indicate the amount of total phenols in mg GAE per L and per g of phenolic compounds extracts, respectively. In the last column, the results are expressed in mg GAE per g of dry mushroom.

Table 6 - Absorbance values and concentrations of total phenolic compounds of mushroom samples.

Mushroom	Absorbance	mg GAE / L mushroom extract	mg GAE / g extract phenolic compounds	mg GAE / g dry mushroom
BE	0.544 ±0.137	29.03±4.86	27.13 ± 4.67	11.21 ± 1.93
TE	0.213 ± 0.055	14.65 ±1.95	13.69 ± 1.60	5.66 ± 0.66
G. luc	0.506 ± 0.033	27.38 ±1.16	26.57 ± 2.35	10.98 ± 0.97
G. ling	0.492 ± 0.158	26.80 ± 5.61	26.17 ± 7.73	10.81 ± 3.20

BE - *Boletus edulis*; TE - *Tricholoma equestre*; G. luc - *Ganoderma lucidum*; G. ling - *Ganoderma lingzhi*
Data are expressed as mean ± standard deviation (n=3). Used dried medicinal mushrooms (1 mg).

The results in Table 6 and Figure 18 show that the mushrooms with higher phenolic content are the *Boletus edulis*, followed by *Ganoderma lucidum*, *Ganoderma lingzhi*, and *Tricholoma equestre.* The three mentioned species have similar phenolic contents that are about two times larger than that of *Tricholoma equestre*.

The *Tricholoma equestre* is the mushroom species that presents the lowest value, with 13.69 ± 1.61 mg GAE / g of mushroom extract *Tricholoma equestre*, while phenolic content in dry *Tricholoma equestre* is 5.66 ± 0.67 mg GAE / g of dried mushroom.

As regards the phenolic content of *Boletus edulis*, the results are similar to those of Vamanu and Nita (2013) who reported values of 21.32 ± 3.78 mg GAE / g of extract, using the same solvent, but different extraction conditions. The total phenolic content in ethanolic extracts of *Ganoderma lucidum* and *Ganoderma linghzi* showed a similar antioxidant potential to that of previously studied extracts. Commercial strain contained of 33.42 GAE / g of mushroom extract (Ćilerdžić *et al.*, 2014). In another study, Heleno *et al*. (2012), using methanolic extracts, found in the fruiting body of *Ganoderma lucidum* a total phenolics content of 28.64 mg GAE / g of extract

In Figure 19, are presented the total phenol content in the different mushrooms extracts and dried mushrooms, whose the amount of total phenols is expressed in mg GAE per g of phenolic compounds extracts and in mg GAE per g of dry mushroom, respectively.

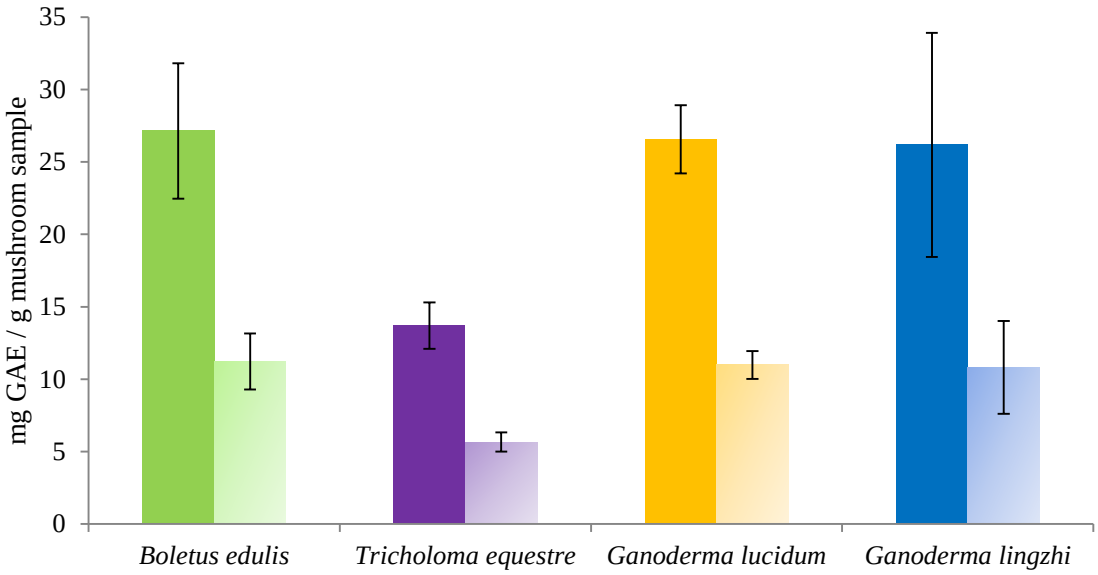
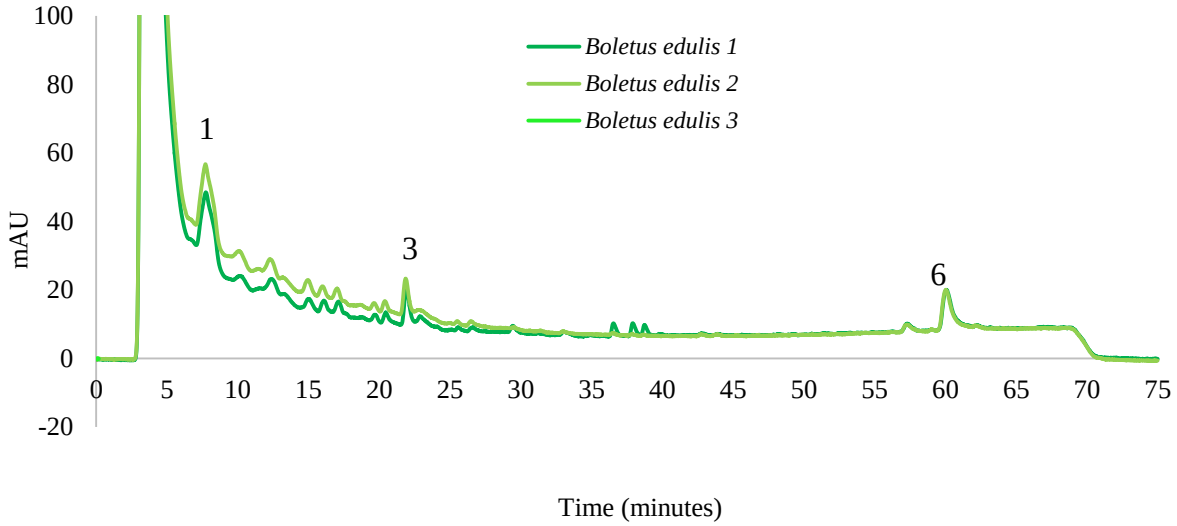


Figure 19 - Total phenol content in different mushroom extracts (darker bars) and in dried mushrooms (lighter bars). Data were expressed as mean ± standard deviation (n=3).

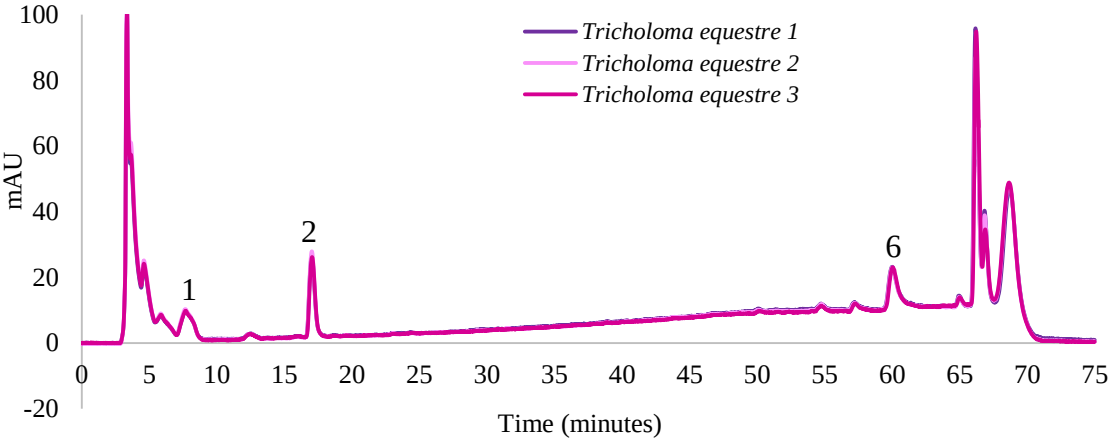
IV. 1.3. HPLC Analysis

HPLC chromatograms at 280 nm of samples of mushroom extracts containing phenolic compounds are presented in Figure 20.

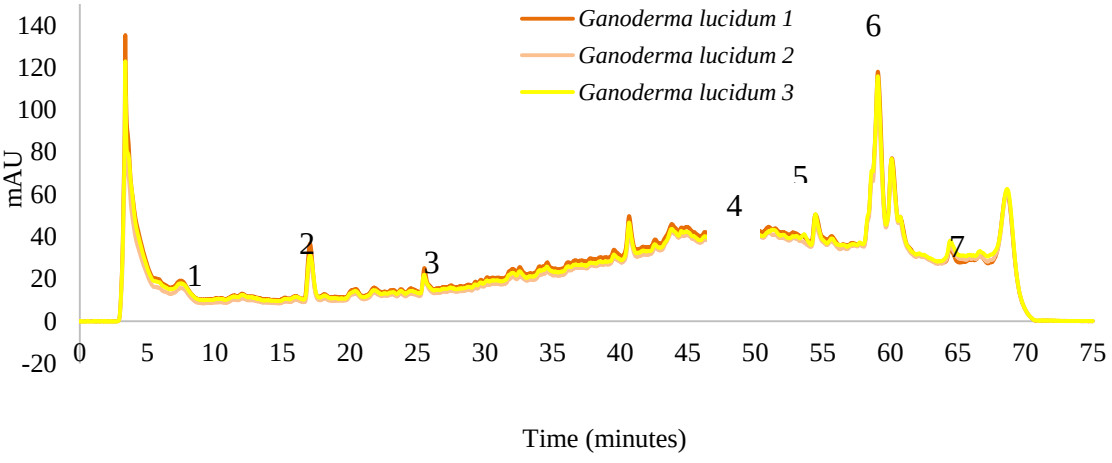
a


Boletus edulis 1
Boletus edulis 2
Boletus edulis 3
mAU
1
3
6
Time (minutes)

b


Tricholoma equestre 1
Tricholoma equestre 2
Tricholoma equestre 3
mAU
1
2
6
Time (minutes)

c


Ganoderma lucidum 1
Ganoderma lucidum 2
Ganoderma lucidum 3
mAU
1
2
3
4
5
6
7
Time (minutes)

d

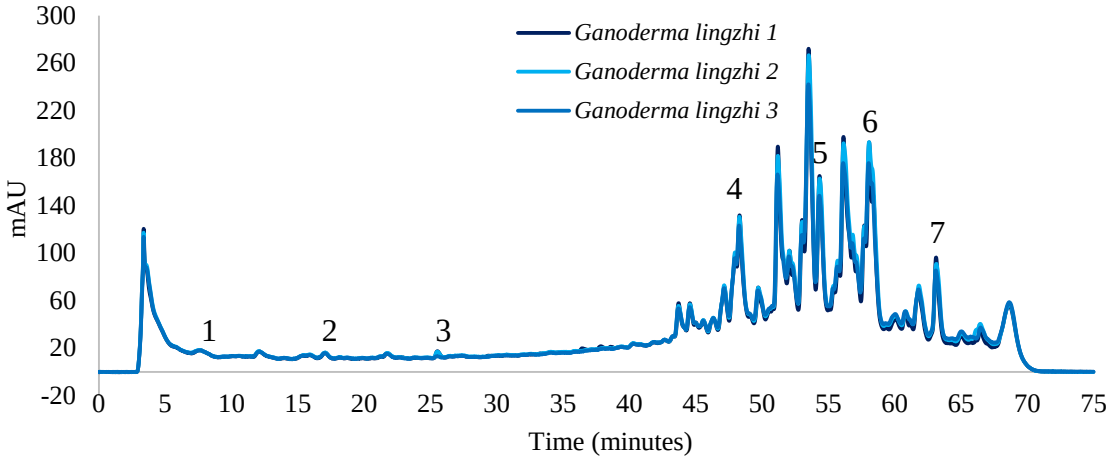


Figure 20 - Representative high performance liquid chromatography (HPLC) profile of mushroom samples. **a.** *Boletus edulis* (n=3). **b**. *Tricholoma equestre* (n=3). **c**. *Ganoderma lucidum* (n=3). ***d.*** *Ganoderma lingzhi* (n=3). Each panel shows superimposed curves of three samples (1, 2, 3), corresponding the first peak (truncated in panel **a**) to the mobile phase injection. The UV/VIS detection was made at 280 nm.

In all chromatograms, it is observed that the chromatographic profiles are coincident for all triplicate analysis of each mushroom. In all cases, the first peak corresponds to the mobile phase injection, being truncated in panel a.

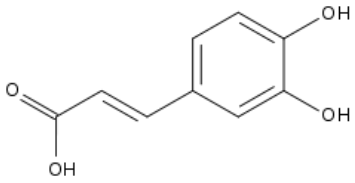
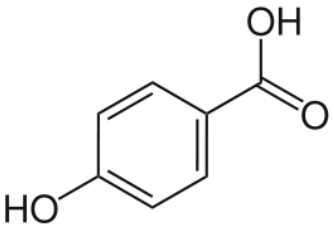
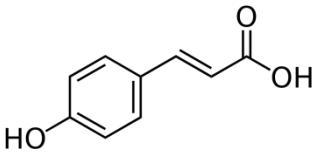
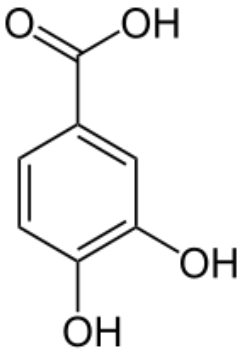
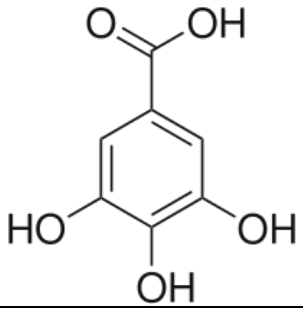
In Figure 20, it is possible to observe peaks that are common to all mushrooms, namely, the peaks 1 and 6. However, the peak 2 is only present in the *Tricholoma equestre*, *Ganoderma lucidum* e *Ganoderma lingzhi* mushrooms. There are also some peaks that are specific to the *Ganoderma* species, of which the peaks 3, 4, 5, 6 and 7 stand out.

Some of these peaks can be associated of a diverse phenolic compounds as p-hydroxybenzoic acid, protocatechuic acid, gallic acid, cinnamic acid, p-coumaric acid caffeic acid that are found frequently in mushrooms.

However more work would be necessary to associate each point to one phenolic compound (identify and quantify the phenolic compounds present in these mushrooms samples) with precision.

Several authors have identified and quantified using HPLC, some phenolic compounds that are typically to found in these mushrooms. Coelho *et al.* (2014) identified some phenolic compounds, using similar condition operations, whose respective retention times and chemical structures are presents in Table 7.

Table 7 - Phenolic compounds and respective chemical structures and retention times (from Coelho *et al.*, 2014).

Phenolic compound	Chemical structure	Retention time (min)
caffeic acid		17
p- hydroxybenzoic acid		21
p-coumaruic acid		24
protocatechuic acid		26
gallic acid		29

IV. 2. Polysaccharide Extracts

IV. 2.1. Yield of polysaccharide extraction process

Table 8 presents the mushroom dried, AIR and WSB weights and respective yields of resulting from the extraction process of polysaccharides for the different strains of mushrooms.

Table 8 - Samples mushrooms dried, AIR and WSB weights and respective yields of extraction process from four mushrooms.

Mushroom	Mushroom dried (g)	AIR (g)	Yield % AIR	WSB (g)	Yield % WSB
BE	25.00	12.20	48.79	0.7061	5.79
TE	50.00	25.80	51.60	0.5979	2.32
G. luc	25.00	19.57	78.28	0.1071	0.55
G. ling	25.00	19.52	78.08	0.0920	0.47

BE -*Boletus edulis*; TE - *Tricholoma equestre*; G. luc -*Ganoderma lucidum*; G. ling - *Ganoderma lingzhi*

The AIR, (an intermediate compound) constituted approximately 49 % of the mass of the mushroom for the species *Boletus edulis*, 52 % for *Tricholoma equestre* and 78 % for the species *Ganoderma lucidum* and *Ganoderma lingzhi*. Regarding the water soluble material, after dialysis (WSB), very low yields of less than 6 % were obtained in all cases.

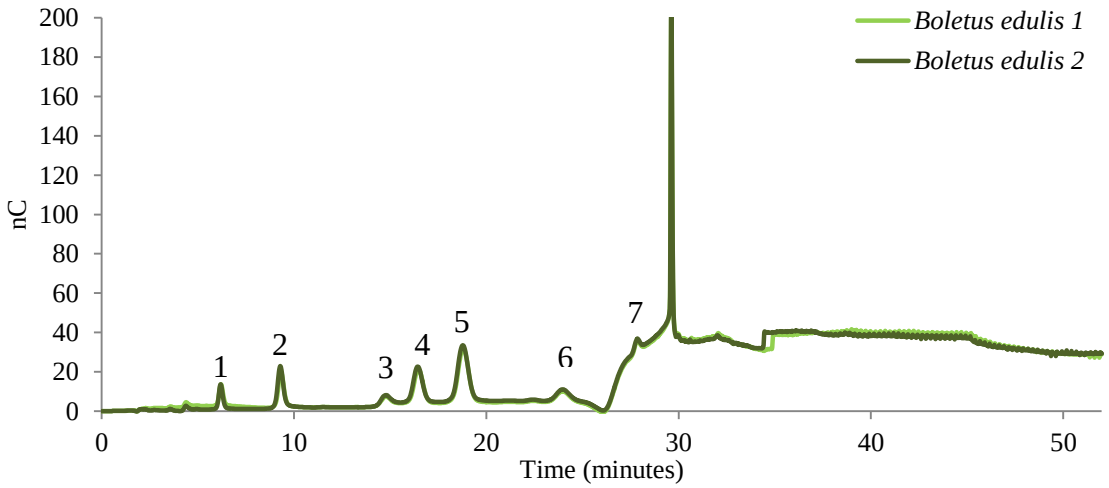
In particular, mushrooms of the *Ganoderma* species show similar yields, and although higher yields were obtained in the AIR, about 78 %, in the WSB, the lowest yields were obtained, close to 0.5 %.

It should be mentioned that all WSM samples, before the dialysis, were solutions with high viscosity and a color ranging from yellow to brown.

IV. 2.2. Analysis of sugars composition

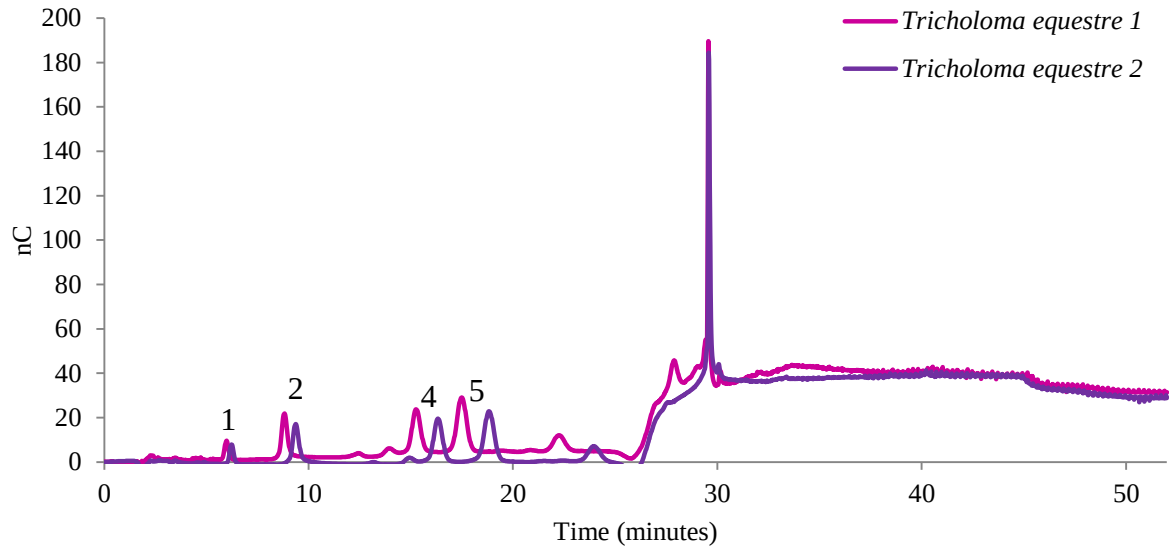
For identification and quantification of the sugar content, the analyzes were carried out in duplicate to ensure replicability of the results. In Figure 21, are compiled the ionic chromatograms for the analysis of the content and nature of the sugars present in WSB samples.

a



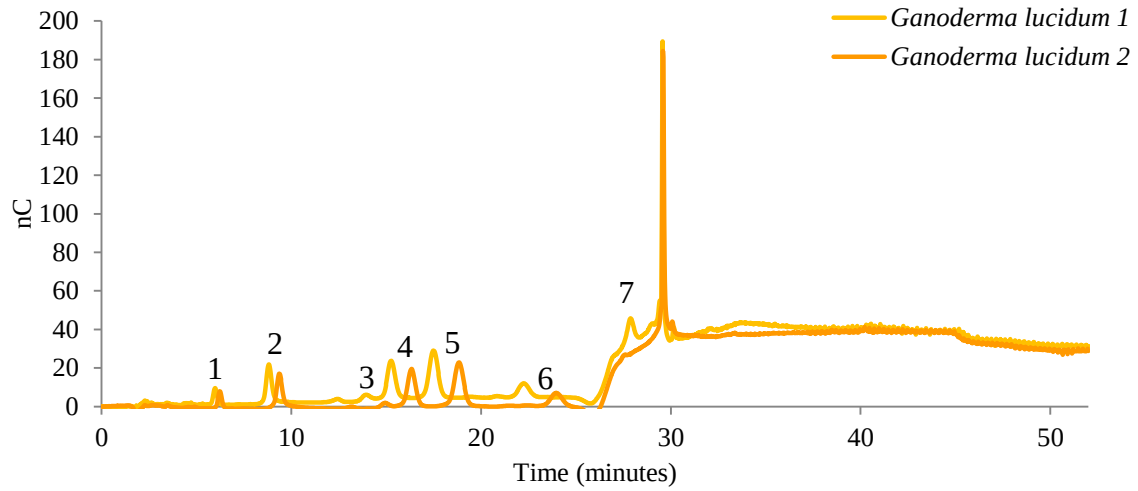
1 - Fucose; 2 - Internal standard; 3 - Glucosamine; 4 - Galactose; 5 - Glucose; 6 - Mannose; 7 - Ribose.

b



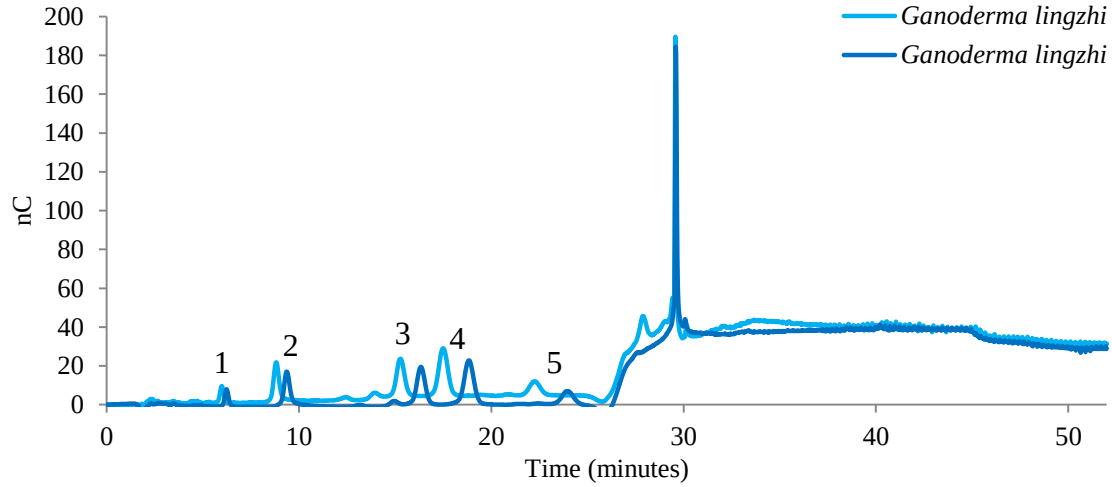
1 - Fucose; 2 - Internal standard; 4 - Galactose; 5 - Glucose.

c



1 - Fucose; 2 - Internal standard; 3 - Glucosamine; 4 - Galactose; 5 - Glucose; 6 - Mannose; 7 - Ribose.

d



1 - Fucose; 2 - Internal standard; 3 - Glucosamine; 4 - Galactose; 5 - Glucose.

Figure 21 - Ionic chromatogram profile of samples of mushrooms. **a**. *Boletus edulis* (n=2). **b**. *Tricholoma equestre* (n=2). **c**. *Ganoderma lucidum* (n=2). **d**. *Ganoderma lingzhi* (n=2).. Each panel shows superimposed curves of two samples (1, 2) for each mushroom.

The sugar identification in samples mushroom was made by comparing the retention times of sample peaks with standards, whose approximated values are presented in Table 8.

As can be seen in Figure 21, the mushrooms species present a variety of sugars, including, Rhamnose, Fuccose, Glucosamine, Galactose, Glucose, Mannose and Ribose, although, the monosaccharides mentioned above are not always present in all mushrooms. As can be seen in Figure 21, were obtained similar results between samples, however in panels B-D, the ionic chromatograms of the two samples are a little dislocated.

For determination of content of sugars present in polysaccharides extracts, WSB, using standard curves of respective sugars analysed (by internal normalization of the chromatographic peak area), whose equations correspondents are presents in Table 9. The equations are equations polynomial of second degree that pass in origin, where X means the ratio between the area of the respective sugar and the area of the sugar used as the internal standard (2-deoxy-D-glucose) and Y the amount of sugar present in mushrooms, in mg.

The content of Fucose present in mushrooms was made using the same equation found to Rhamnose; analogous situation it happened with Glucosamine that use the same equation found to Glucose since the respective sugars were not available to make their calibration curve.

Table 9 - Equations of standard curves for determination of content of sugars present in polysaccharides extracts and respective values of time retention of these sugars.

Sugar	Equation	R^2	Time retention (min)
Fucose	$Y = 0.196\ X^2 + 0.176\ X$	0.996	6.2
Internal standard	----	----	9.3
Rhamnose	$Y = 0.196\ X^2 + 0.176\ X$	0.996	13.2
Glucosamine	$Y = -0.034\ X^2 + 0.192\ X$	0.981	14.8
Galactose	$Y = -0.018\ X^2 + 0.435\ X$	0.992	16.4
Glucose	$Y = -0.034\ X^2 + 0.192\ X$	0.981	18.8
Mannose	$Y = 0.045\ X^2 + 0.257\ X$	0.980	23.8
Ribose	$Y = 0.001\ X^2 + 0.026\ X$	0.997	27.7

The results of sugars content present in mushrooms are presented in Table 10 and are expressed in mg of sugar per 100 g mushroom dry. It is noted that the portion of Fucose and Rhamnose are presented together in Table 10, since it was not possible to determine the individual amounts of these present in the mushrooms.

Table 10 - Sugar content in mg of sugar for each 100 g of mushroom dry.

Mushroom Sugar	*BE*	*TE*	*G. luc*	*G. ling*
Rhamnose/Fucose	0,4136 ± 0,03	0,3864 ± 0,02	0,2843 ± 0,01	0,1951 ± 0,00
Glucosamine	0,2364 ± 0,03	0	0,1274 ± 0,02	0,2084 ± 0,00

Galactose	2,2954 ± 0,09	1,5769 ± 0,09	2,1360 ± 0,21	1,7099 ± 0,01
Glucose	1,0766 ± 0,01	0,4321 ± 0,02	0,9519 ± 0,04	1,0734 ± 0,00
Mannose	0,5820 ± 0,07	0	0,6014 ± 0,01	0
Ribose	0,0268 ± 0,00	0	0,0057 ± 0,01	0
Total amount	4,6307 ± 0,23	2,3954 ± 0,13	4,1067 ± 0,32	3,1869 ± 0,01

Table 10 show that the most abundant sugar are the Galactose that represents 50 %, 67 %, 52 % and 54 %, of total sugars analysed in *Boletus edulis*, *Tricholoma equestre*, *Ganoderma lucidum* and *Ganoderma lingzhi*, respectively. Other sugar present in significant amounts was Glucose, where it represents 17 %, 23 %, 23 % and 34 % of the sugars present in *Tricholoma equestre*, *Boletus edulis*, *Ganoderma lucidum* and *Ganoderma lingzhi*, respectively.

There were also found Rhamnose and / or Fucose and Glucosamine in smaller amounts in all mushrooms samples, while was only found trace amounts of Ribose in *Boletus edulis* and *Ganoderma lucidum*. This sugar, as well as Mannose, were not found in the *Tricholoma equestre* or *Ganoderma lingzhi*.

The mushrooms *Boletus edulis* have the highest content in total sugars, presenting 4.63 mg of sugars in each 100 g of mushroom dry, followed by *Ganoderma lucidum*, with 4.11 mg of sugars / 100 g mushroom dry. Regarding *Tricholoma equestre*, this presents the lowest sugar content, containing only 2.39 mg / 100 g mushroom dry.

There are many species of mushrooms that contains a variety of sugars (carbohydrates), of which Glucose and Rhamnose as the principal sugars. However, other sugars, like as Xylose, Mannose, Galactose and Fructose are also detected in trace amounts in species mushrooms (Sharma and Gautam, 2015).

In the case of *Ganoderma* species (e.g., *G. lucidum*), Glucose is usually the major sugar component (Wang et al., 2002), but it also exist small amounts of other sugars, such as Mannose, Fucose, Galactose, Xylose, Rhamnose and Fructose (Wang *et al.*, 2002; Ferreira *et al.*, 2015).

Regarding the B*oletus edulis* mushrooms, it is also verified the presence of Glucose, Mannose, Galactose, Xylose and Rhamose (of which the monosaccharides Glucose and Mannose are present in higher amounts, however this is not always verified) (Luo *et al*., 2012).

According to Heleno *et al*. (2015), the presence of Glucose was verified with a content of 1.24 g / 100g of mushroom dry, in *Boletus edulis*. However, in another study it was evaluated the sugar composition of ethanolic extracts, using different conditions and found a value of 370 mg of glucose / 100 g of mushroom *Boletus edulis* dry (Jedidi *et al.*, 2016). These values are significantly greater when compared with the results obtained in this work.

In another study, Heleno *et al.* (2012) used ethanolic extracts prepared from *Ganoderma lucidum* mushrooms, but in different conditions (and reagents) for determination of content of sugars, it was found 0.55 g of glucose in 100 g of mushroom dry, a much higher value when compared with the results obtained in this study.

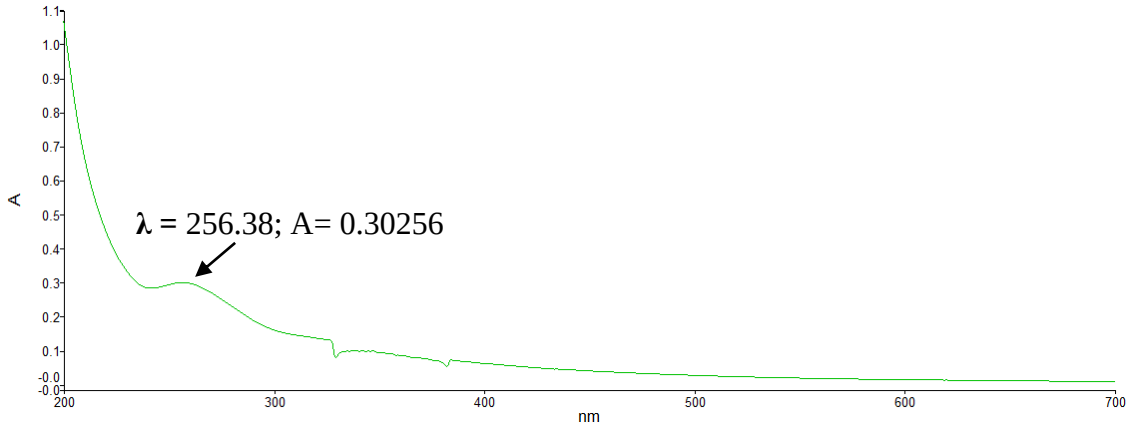
The content of the sugars evaluated in this analysis is quite low, although these are the most common sugars found in mushrooms. However, these results do not mean that mushrooms cannot contain others sugars than those that were accounted for in this analysis.

IV. 2.3. RNA

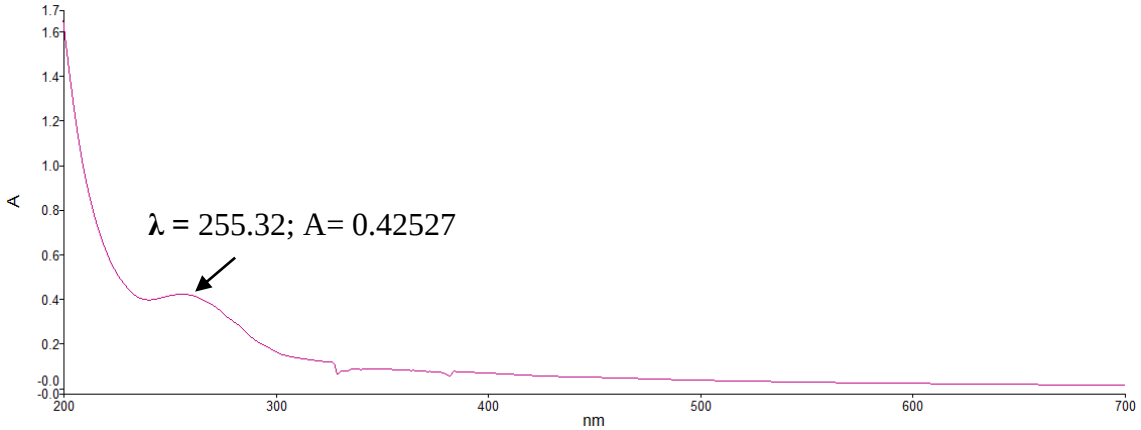
Through the analysis of the sugars, it was found that some of mushrooms studied contained small amounts of ribose. Taking into account previous studies the presence of this monosaccharide suggests that nucleic acid, RNA, is present in the mushrooms. Therefore, in order to verify its presence, a spectral scanning was performed in the UV / VIS region.

In Figure 22 are presented the resulting spectra and respective values of the wavelength (λ, in nm) correspondents to the maximum absorbance (A).

a



b



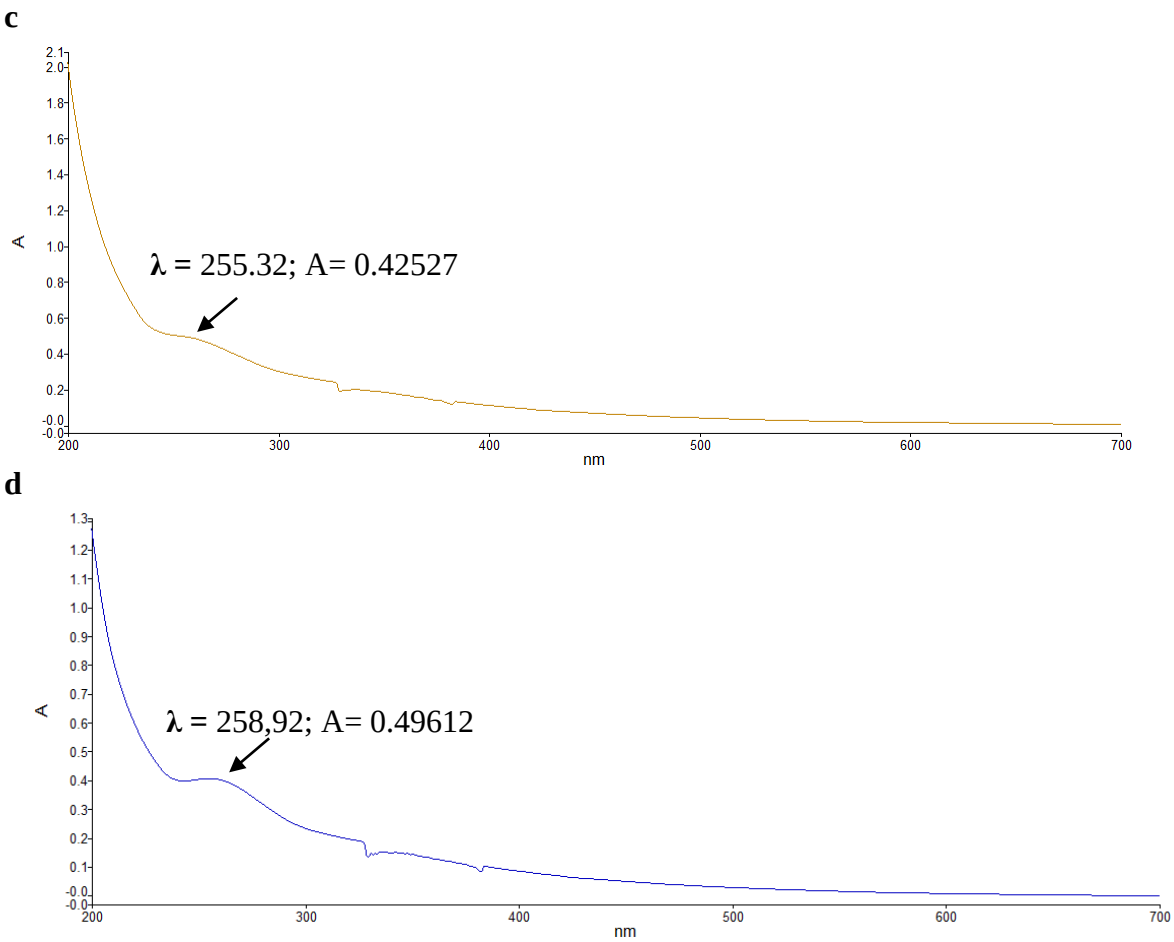


Figure 22 - UV-Vis spectra of extracts polysaccharides from the mushrooms. **a**. B*oletus edulis*. **b**. *Tricholoma equestre*. **c.** *Ganoderma lucidum*. **d**. *Ganoderma lingzhi*.

As can be seen in the Figure 22, the UV-VIS spectra and the wavelength obtained are typical of presence of nucleic acids, since that the maximum absorption found was close to 257 nm. This is the wavelength that indicates the presence of RNA in mushrooms.

Despite of the fact that ribose was not found in mushrooms *Ganoderma lingzhi* and *Tricholoma equestre*, when sugars were quantified, the UV-Vis spectra revealed the presence of RNA.

In relation to the UV-Vis spectrum corresponding to the mushroom *Ganoderma lucidum*, this shows a poorly defined peak which is in agreement with the results obtained in the quantification of ribose, in which trace amounts of this monosaccharide were found. The UV-Vis spectra profile of *Boletus edulis* evidences the presence of RNA, which is also in line with the results obtained in the quantification of sugars, since this mushroom is the one with the highest ribose content.

IV. 3. Elemental analysis

Figure 23 shows the region of the dried mushroom sample that was subjected to X-ray analysis. These images, have been extended 26 times and were obtained with a CCD camera, during analysis moment, after being focused.

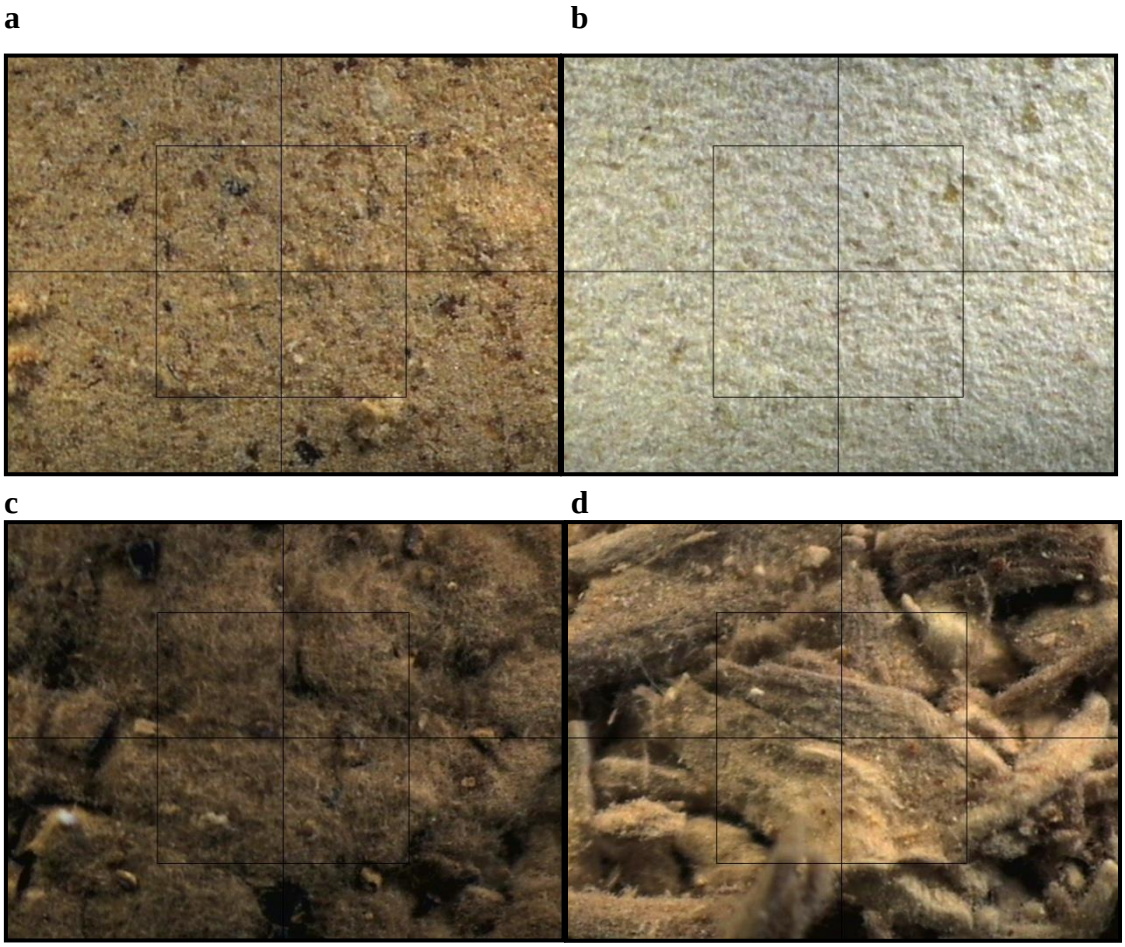


Figure 23 - Region of lyophilized mushroom sample subjected to analysis. **a**. *Boletus edulis*. **b**. *Tricholoma equestre.* **c**. *Ganoderma lucidum.* **d**. *Ganoderma lingzhi.*

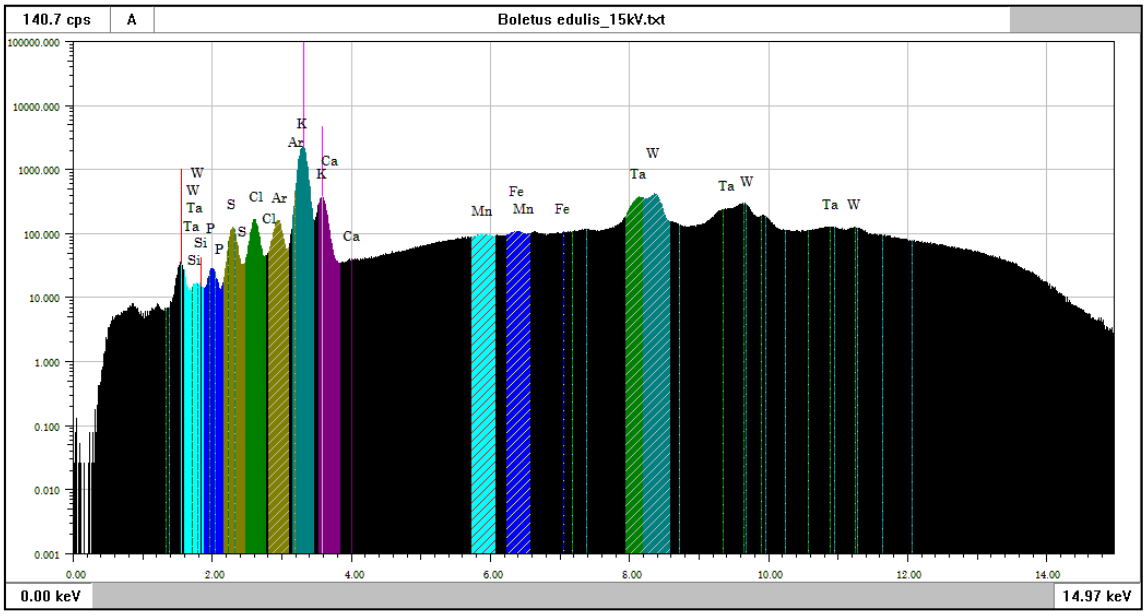
Figure 23 gives an idea of the appearance of the mushrooms, after the grinding process. According to the images, B*oletus edulis* (Figure 23a) and *Tricholoma equestre* (Figure 23b), mushrooms present a more uniform and compact appearance similar to a powder, whereas the *Ganoderma* species mushrooms (Figures 23c and 23d) show a more woody and spongy appearance.

Spectra resulting from the analysis are presented in Figure 24, in which it is possible to identify the elements since that each peak corresponds a chemical element. It should be noted that on the Y-axis, the units are "counts per second" (Logarithmic scale, for better perception and visualization of peaks) and on the X-axis is energy in keV.

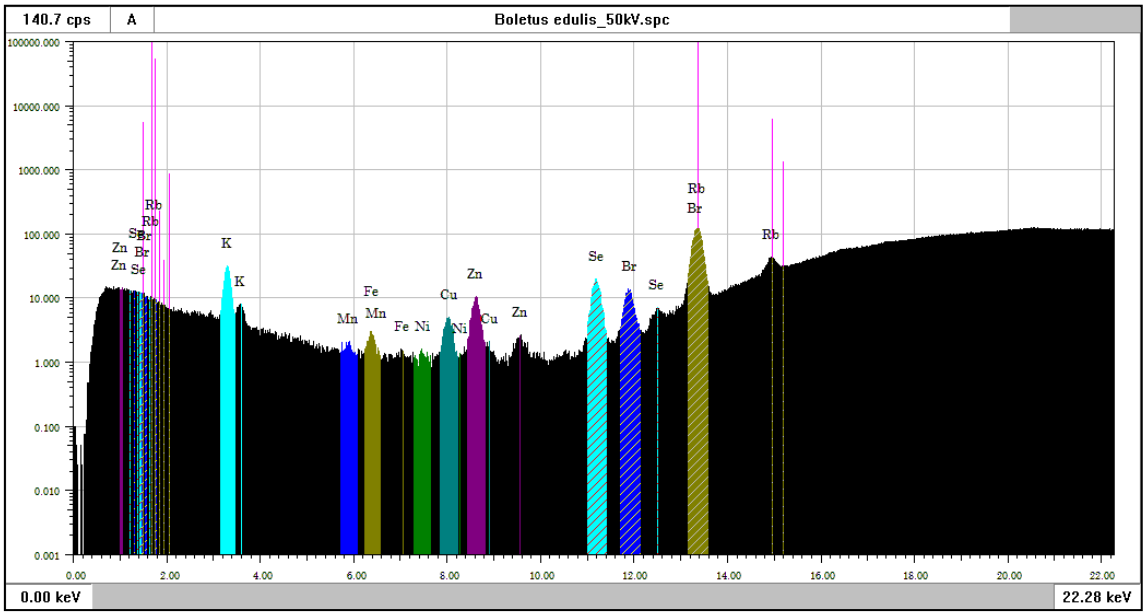
It should be noted that the position of the elements is characteristic of these elements, not depending on the sample concerned.

The larger colored bands correspond to more probable electronic transitions and therefore have a larger number of counts. However, there are also visible lines of the same color as the bands that are displaced corresponding to less probable transitions of the electrons belonging to the chemical elements and therefore have a lower intensity.

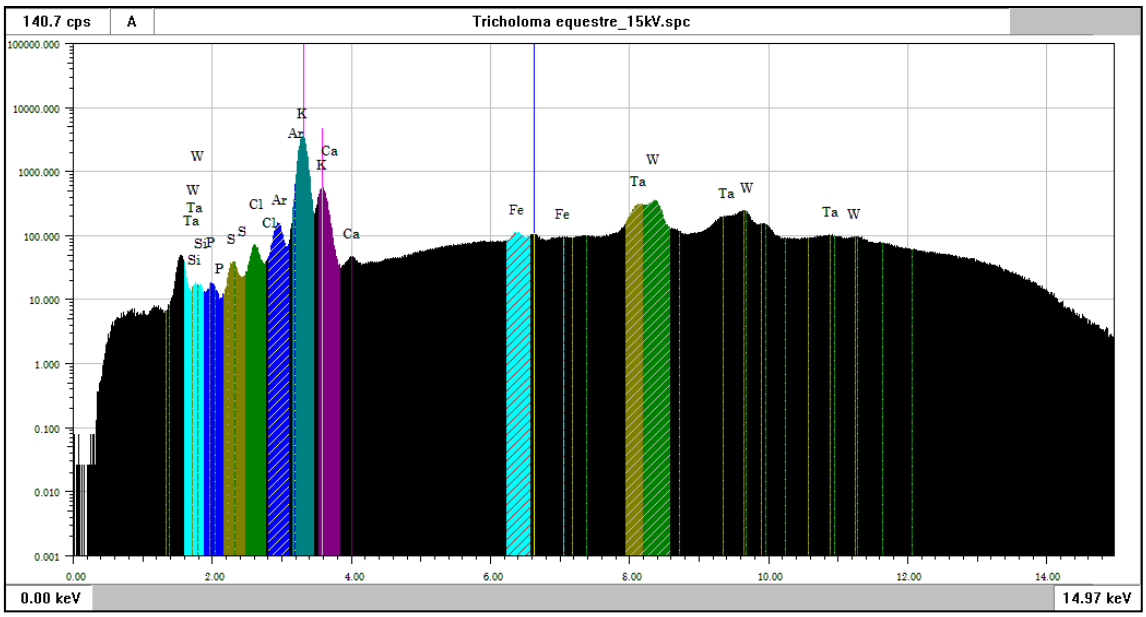
a.1



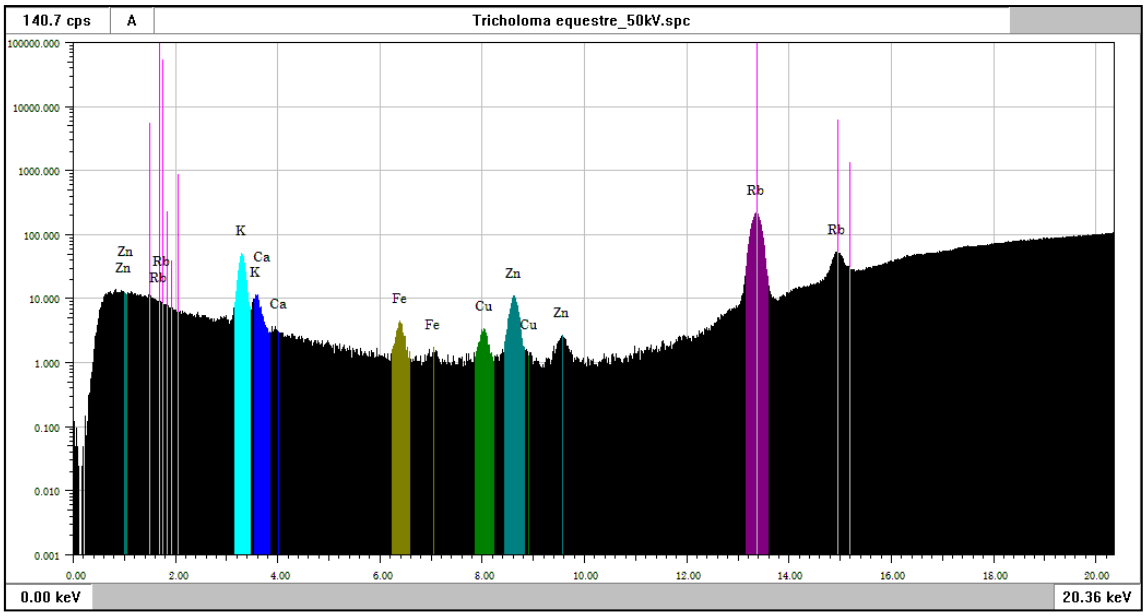
a.2



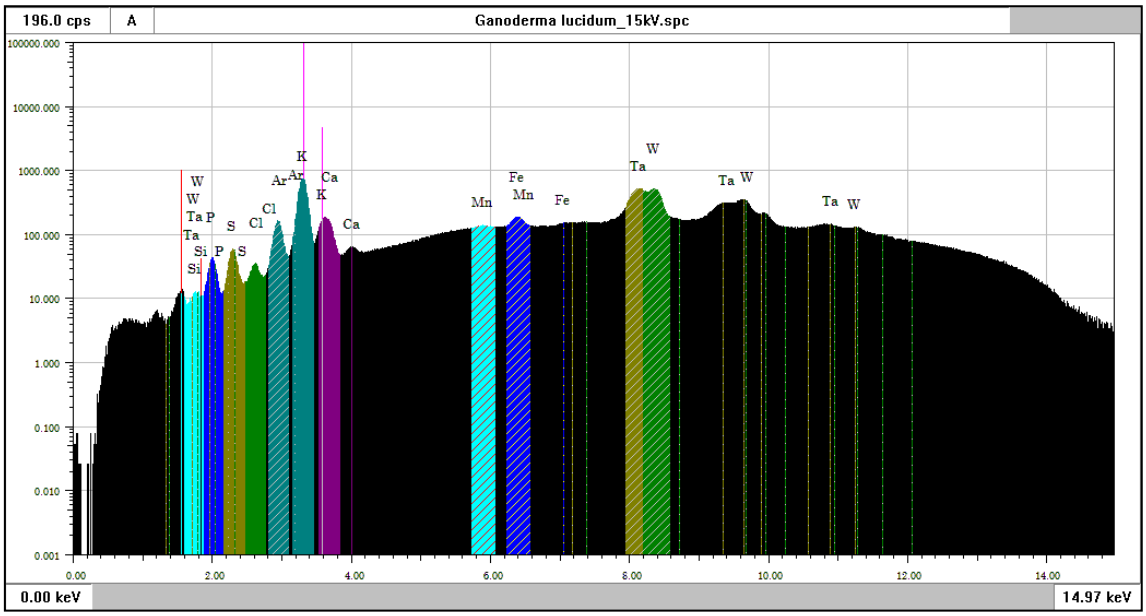
b.1


140.7 cps
A
Tricholoma equestre_15kV.spc
K
Ar
Ca
K
W
W
Ta
Ta
Si
Si
P
P
S
S
Cl
Cl
Ar
Ca
Fe
Fe
W
Ta
Ta
W
Ta
W
0.00 keV
14.97 keV

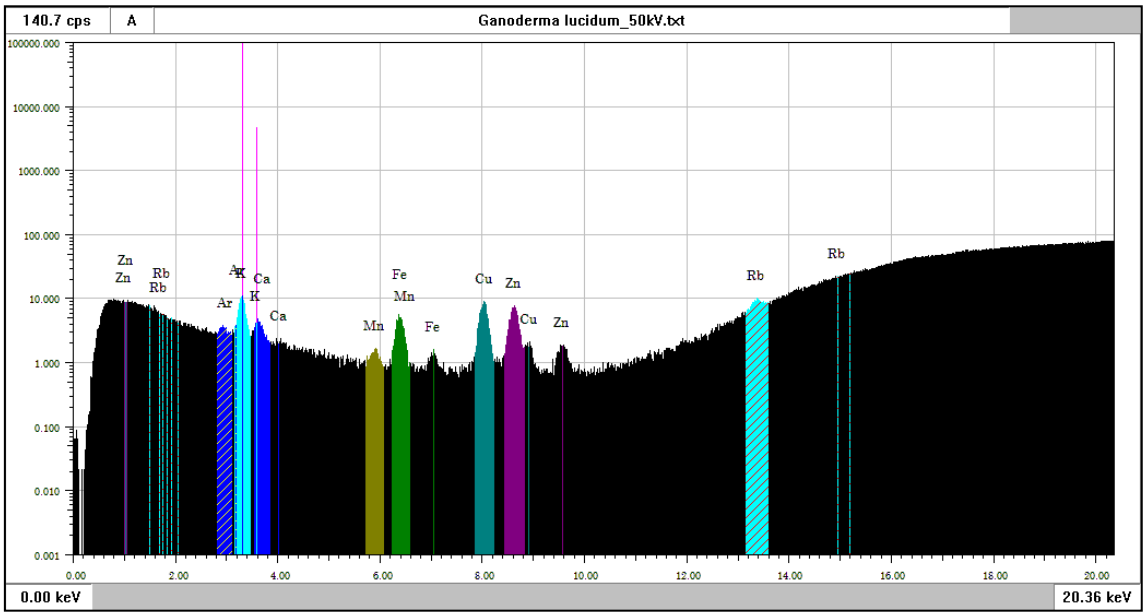
b.2


140.7 cps
A
Tricholoma equestre_50kV.spc
Zn
Zn
Rb
Rb
K
Ca
K
Ca
Fe
Fe
Cu
Cu
Zn
Zn
Rb
Rb
0.00 keV
20.36 keV

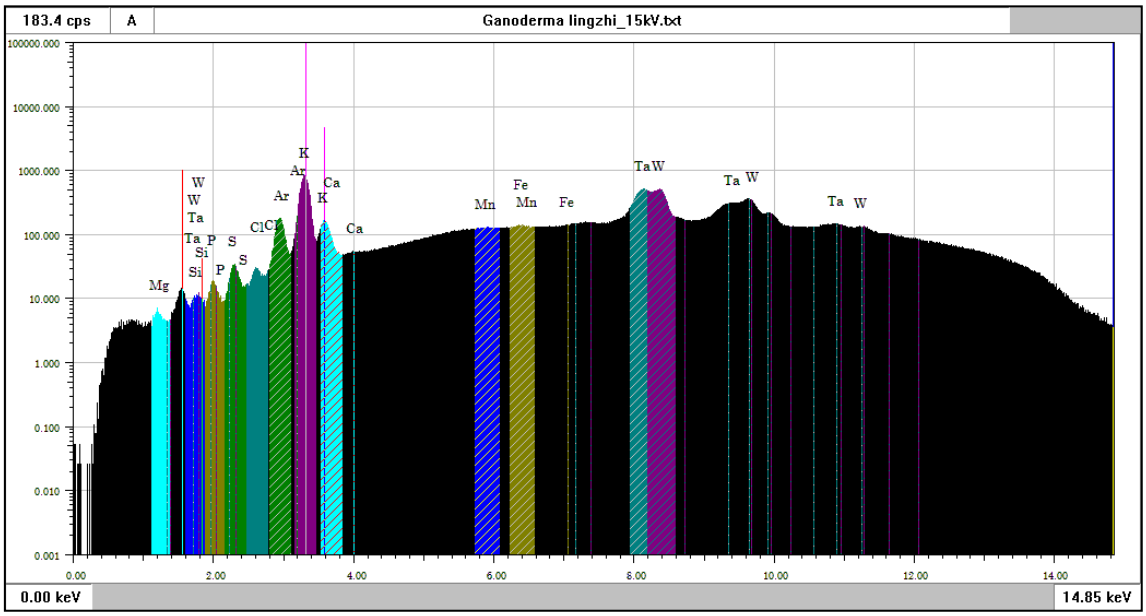
c.1


196.0 cps
A
Ganoderma lucidum_15kV.spc
0.00 keV
14.97 keV

c.2


140.7 cps
A
Ganoderma lucidum_50kV.txt
0.00 keV
20.36 keV

d.1


183.4 cps
A
Ganoderma lingzhi_15kV.txt
0.00 keV
14.85 keV

d.2

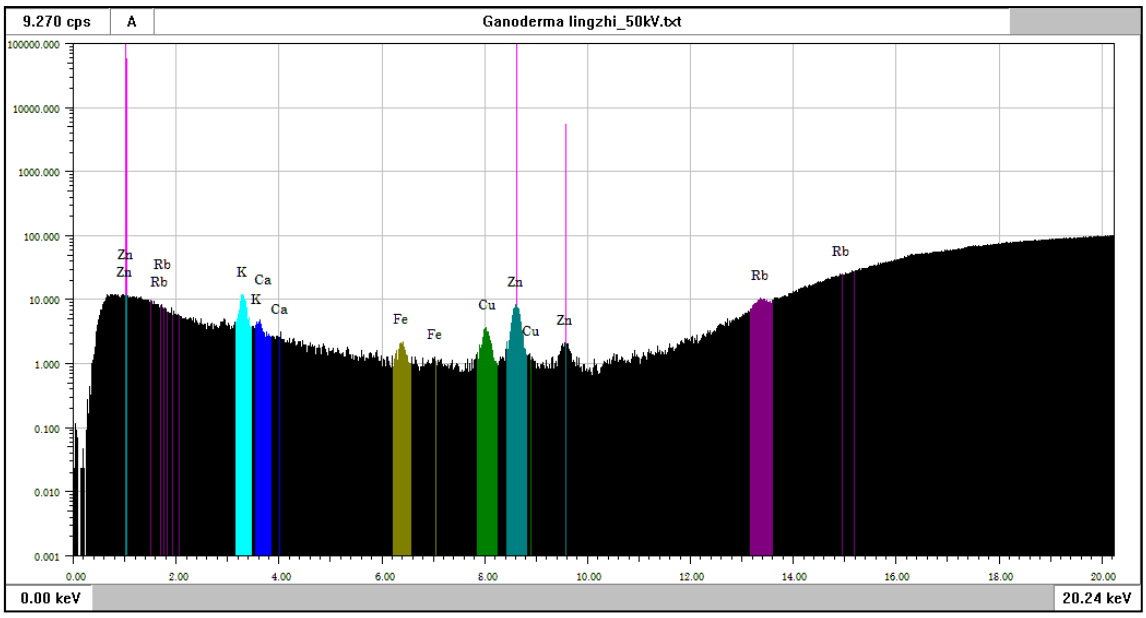


Figure 24 - Spectra resulting from the chemical analysis of mushrooms using XRF. **a.1.** *Boletus edulis* (15 keV); **a.2.** *Boletus edulis* (50 keV); **b.1.** *Tricholoma equestre* (15 keV); **b.2.** *Tricholoma equestre* (50 keV); **c.1.** *Ganoderma lucidum* (15 keV); **c.2.** *Ganoderma lucidum* (50 keV); **d.1.** *Ganoderma lingzhi* (15 keV); **d.2.** *Ganoderma lingzhi* (50 keV).

According to the XRF analyzes, several chemical elements could be identified in the mushrooms samples, where 15 (fifteen) elements are described: *Mg*, *Si*, *P*, *S*, *Cl*, *K*, *Ca*, *Mn*, *Fe*, *Ni*, *Cu*, *Zn*, *Se*, *Br* and *Rb*.

It should be noted that the spectra also show the presence of other three chemical elements (*Ta*, *W*, *Ar*), which are not actually present in the mushrooms, but which serve to explain some peaks that are consequent of the conditions under which the analyzes were carried out.

Table 11 shows the metal elements present in all four dried mushrooms samples.

Table 11 - XRF identification of chemical elements present in mushrooms.

Z	Element	Element Name	BE	TE	G. luc	G. ling
12	Mg	**Magnesium**	✗	✗	✗	✓ 1
14	Si	**Silicon**	✓ 1	✓ 1	✓ 1	✓ 1
15	P	**Phosphorus**	✓ 1	✓ 1	✓ 1	✓ 1
16	S	**Sulphur**	✓ 1	✓ 1	✓ 1	✓ 1
17	Cl	**Chlorine**	✓ 1	✓ 1	✓ 1	✓ 1
19	K	**Potassium**	✓ 1,2	✓ 1,2	✓ 1,2	✓ 1,2
20	Ca	**Calcium**	✓ 1	✓ 1,2	✓ 1,2	✓ 1,2
25	Mn	**Manganese**	✓ 1,2	✗	✓ 1,2	✓ 1
26	Fe	**Iron**	✓ 1,2	✓ 1,2	✓ 1,2	✓ 1,2
28	Ni	**Nickel**	✓ 2	✗	✗	✗
29	Cu	**Copper**	✓ 2	✓ 2	✓ 2	✓ 2
30	Zn	**Zinc**	✓ 2	✓ 2	✓ 2	✓ 2
34	Se	**Selenium**	✓ 2	✗	✗	✗
35	Br	**Bromine**	✓ 2	✗	✗	✗
37	Rb	**Rubidium**	✓ 2	✓ 2	✓ 2	✓ 2

BE - *Boletus edulis*; TE - *Tricholoma equestre*; G. luc - *Ganoderma lucidum*; G. ling - *Ganoderma lingzhi.*
Chemical element detected using low-intensity energy, 15 KeV ([1]) and / or high intensity energy, 50 KeV ([2]).
Chemical element present (✓) or not present (×) in mushroom.

In Table 11, it is possible to observe several chemical elements in different mushrooms samples, namely silicon, phosphorus, sulfur, chlorine, potassium, calcium, iron, copper, zinc and rubidium. However, there are other chemical elements that are present only in some types of mushrooms, such as nickel, selenium and bromine, present only in the mushrooms *Boletus edulis*, and magnesium that is unique to *Ganoderma lingzhi*.

In relation to the species *Ganoderma*, they apparently contain the same chemical elements in their constitution, since only the mushroom *Ganoderma lingzhi* contains an additional element, the magnesium. This element, however, was not found in any other mushroom. So, through XRF analysis it is not possible to distinguish the two species *Ganoderma*.

In a study, carried out by Carvalho *et al.* (2005) also revealed the presence of metals such as *K*, *Ca*, *Mn*, *Fe*, *Cu*, *Zn*, *Rb* and *Pb* in *Tricholoma equestre* collected near a high traffic road in Porto and in mushrooms *Boletus edulis* harvested in a clean wooded region.

The mushroom *Ganoderma lucidum* was also the target of the chemical composition analysis, performed by Chan and Lo (2003), using other equipments. However, authors identified the presence of elements such as *Mg*, *P*, *K*, *Ca*, *Fe*, similar to the results obtained in this study, but also others elements, some of them that are dangerous and toxics as *Na*, *Al*, *Ba* and *V*, *As*, *Se*, *Cd*, *Hg* and *Pb*, in trace amounts.

In this present work, no trace of the presence of *Pb*, *Cd* or *Hg* was identified in the analyzed mushrooms, which indicates safety in the products that were harvested. In view of the presence of essential nutrients found in mushroom samples as well as their total phenol content, it is suggested that mushrooms need to be studied in greater depth to explore all strands and maybe they can be used in more different medical applications.

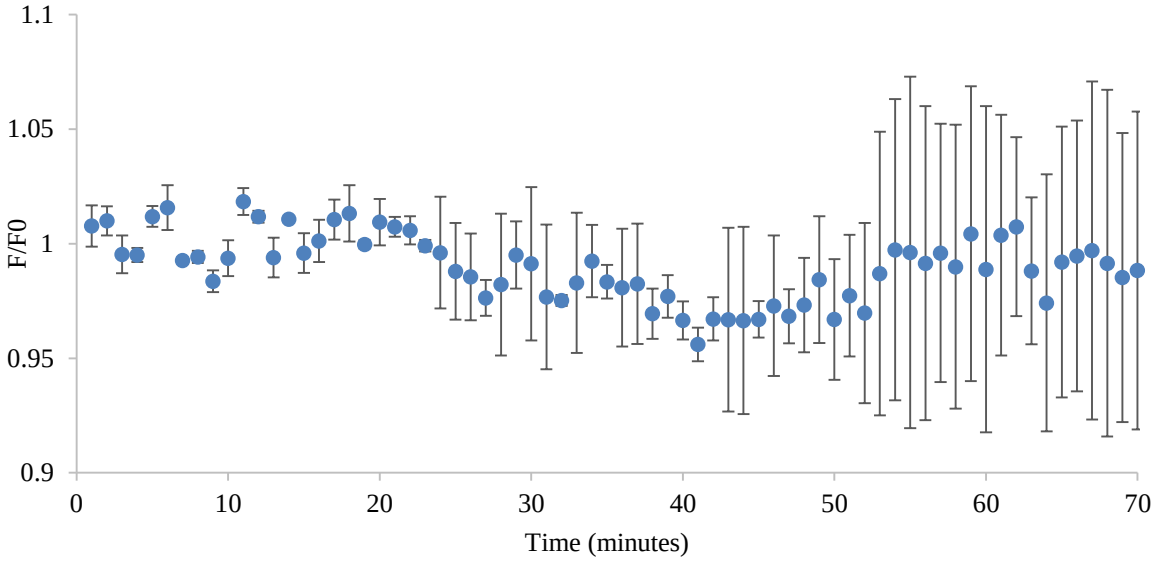
IV. 4. Neuronal ROS studies

In this section, we present the results obtained in the performed experiments that consisted in the detection of optical signals occurred in the synapses of the mossy fibers of the hippocampus CA3 area.

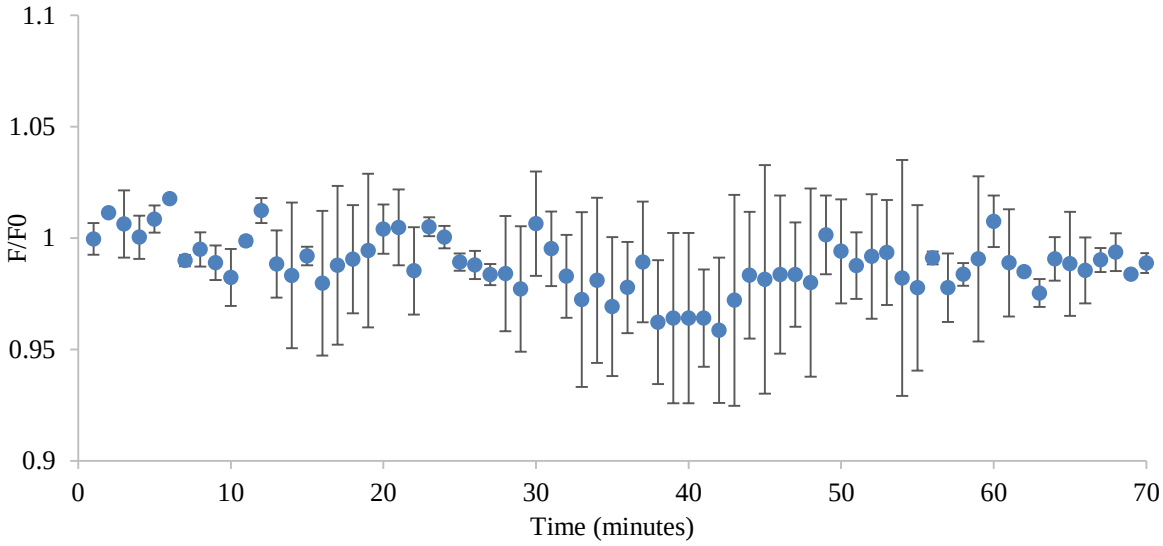
In order to evaluate the effects induced of polysaccharides on the ROS signals, it were tested different concentrations of polysaccharides extract from *Boletus edulis* in hippocampal slices that was previously incubated in a ROS solution for 1 hour.

The results are presents in Figure 23. The hippocampal slice was perfused during the initial 10 minutes with a solution of ACSF, followed by a solution of ACSF to which polysaccharides were added over 30 minutes. At the end of this time, the hippocampal slice was perfused again with a fresh solution of ACSF for another 30 minutes.

a



b



c

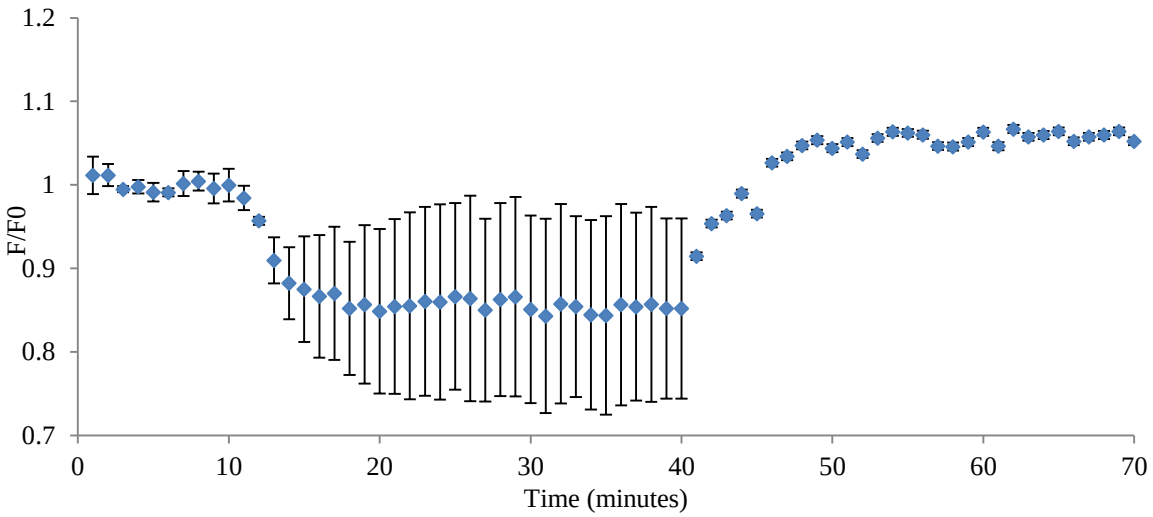


Figure 25 - Effect of various concentrations of polysaccharides extracted from the mushroom *Boletus edulis* on ROS signals. **a.** Signals obtained at a concentration of 0.1 g / L (n = 2). **b.** Signals obtained at a concentration of 0.5 g / L (n = 2). **c**. Signals obtained at a concentration of 1 g / L (n = 4, from t = 0 minutes to t = 40 minutes and n = 2, from t = 40 minutes). Each point is expressed as mean ± standard deviation.

As shown in Figure 25, the concentration of *Boletus edulis* polysaccharides that induce greater variation in ROS signals is 1 g / L, with a depression measuring 14.58 % of control at 35 - 40 min.

Observing the results obtained using a concentration of 1 g/L of polysaccharides (Figure 25c), it is verified that the intensity of the ROS signal decrease very fast, from the moment the solution containing polysaccharides is added (10 min) and maintains stable until it introduces a new solution (t = 40 min). When it returns to the ACSF solution, an increase of the ROS signal is observed that exceeds the baseline, which suggests the existence of a potentiation phase (6.16 %).

In case of Figure 25a, when is introduced a solution with a concentration of 0.5 g / L polysaccharides, it is verified a small decrease of ROS signal of 2.22 % and when the ACSF solution is perfused again, the intensity increases to baseline values, showing an enhancement of 0.6 %. Similar behavior is observed when using a concentration of 0.1 g / L, in which a decrease of 1.73 % was obtained, recovering the signal (0.39 %) following washout.

So, in conclusion, the results suggest that the signal decreases in the presence of polysaccharides, that is, that these compounds attenuate the negative effects of ROS. However, since few experiments were performed, further experiments would be required to more safely conclude that there is a decrease in the formation of ROS in the presence of polysaccharides.

It is also possible to observe that in all experiments, the signal is reversible, indicating that biological preparation was not affected.

V. Conclusions and future work perspectives

The main aim of the present study was to chemically characterize the species of mushrooms *Boletus edulis*, *Tricholoma equestre*, *Ganoderma lucidum* and *Ganoderma lingzhi*.

As for the yield obtained during the extraction process of phenolic compounds, this was higher than 40 %, for the species *Boletus edulis* and *Tricholoma equestre*, whereas in the case of the *Ganoderma* species, this yield presented well below 10 %.

With respect to the phenolic content, it is possible to conclude that the mushrooms *Boletus edulis*, *Ganoderma lucidum* and *Ganoderma lingzhi* presented values of about 27 mg GAE / g of mushroom extract, while that the *Tricholoma equeste* was about half that. It should also be noted that when assessing the phenolic content in dry mushrooms, this performance is also verified, in which *Tricholoma equestre* contains approximately 6 mg GAE / g of dry mushroom.

HPLC tests were also performed to determine the type of phenolic compounds present in the mushrooms, and through the analysis of the chromatograms obtained, it was possible to verify that there are some compounds that are common to several mushrooms, but it was not possible to identify them.

In the extraction of polysaccharides intermediate yields were obtained close to 80% for the *Ganoderma* species, while for the remaining mushrooms these were not far from 50%. The overall yield of the extraction process did not exceed 6%.

As regards the analysis of the composition of sugars, a variety of monosaccharides were found, among them Rhamnosis, Fuccose, Glucosamine, Galactose, Glucose, Mannose and Ribose. Of these, Galactose stands out because it is the monosaccharide present in greater abundance.

Regarding to the total amount of monosaccharides contained in the mushrooms, this is quite small. The mushroom *Boletus edulis* stands out, because it has a higher sugar content, with a content of 4.6 mg of sugars per 100 g of dried mushroom.

The results obtained in this work also allowed to conclude that all the mushrooms have RNA in their constitution.

The analyzes performed through XRF allowed to identify several chemical elements that can be found in the mushrooms. Thus, it was possible to identify about 15 elements which are part of the composition of mushrooms: magnesium, silicon, phosphorus, sulfur, chlorine, potassium, calcium, manganese, iron, nickel, copper, zinc, selenium, bromine and

rubidium. It is emphasized that most of these are essential nutrients and beneficial for health, being completely safe its consumption and use in medical applications.

Taking into account the results obtained previously and considering that it is an edible, safe and pleasant species, it was decided to carry out the experiments of the synaptic analysis, using the extract from mushrooms *Boletus edulis*. It is important to note that the amount extracted from the mushroom was very small, which did not allow further studies in slices.

Regarding the results of hippocampal slice experiments, the results indicate that polysaccharides attenuate the negative effects of ROS. This fact is more evident in the case of using a concentration of 1g / L of polysaccharides. However, it would be pertinent to carry out more experiments to affirm the veracity of these results.

At the end of this study, many issues that stayed to be studied in depth, and new perspectives of future research have emerged. In this context, some suggestions are suggested for future work that will enrich and complete the studies carried out, which are mentioned taking into account the division of work.

At the level of the characterization of the mushrooms, it was of interest to carry out the ABTS technique, using the obtained extracts, in order to allow an evaluation of the antioxidant capacity offered by these mushrooms. With regard to the HPLC technique, and considering that the analyzes carried out did not allow the quantification of the phenolic compounds that are part of the studied mushrooms, as well as the association of each peak to a phenolic compound. Thus, a more detailed analysis is one of the proposals for future work for a complete characterization.

It is proposed as future work, the quantification of the chemical elements identified in the XRF analysis, for a better characterization of these species.

Thus, it is suggested as future work, also the realization of more experiments, using hippocampal slices, under the same conditions, using the extracted / coming from the remaining mushrooms *Tricholoma equestre*, *Ganoderma lingzhi*, *Ganoderma lucidum*. The objective would be to compare the signs obtained in this study and verify if the answers obtained here have any basis. In addition, it is suggested to carry out experiments using the phenolic extracts obtained and to study the effects on synaptic activity, using extracts containing exclusively phenolic compounds or flavonoids.

It would also be interesting to study if the harvesting site of the *Tricholoma equestre* has influence in the concentration of extracted compounds studied. It would also interesting to extract other compounds present in these mushrooms and later to evaluate its impact on synaptic activity.

VI. References

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