UNIVERSIDADE D
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ANALYSIS OF IMPACT ON NEURONAL ROS PRODUCTION

VOLUME 1

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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UNIVERSIDADE DE COIMBRA

Dedico esta tese de Mestrado

às minhas estrelinhas que estão no céu:

Aos meus **pais**: **Fátima** e **António**

e ao meu **irmão Paulo**

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Abstract

Presently, the rapid rate of growth of the affected population with premature aging and numerous diseases is evident being cancer and neurodegenerative diseases, such as Alzheimer's, Parkinson's and amyotrophic lateral sclerosis, the most prevalent and requiring more attention. One of the main causes for the onset of these diseases is the uncontrolled production of reactive oxygen species (ROS), derived from an excessive production of free radicals. In order to reduce oxidative damage and considering the restrictions on the use of synthetic antioxidants, there is an increasing demand for natural products that contain a high antioxidant potential.

Among the existing natural products, mushrooms have been the subject of countless studies, due to their beneficial effects on health in addition to their nutritional properties. These are fungi quite rich in bioactive compounds, such as polysaccharides, phenolic compounds, vitamins and secondary metabolites. Some of the referred compounds have numerous antioxidant properties that are very beneficial to the organism. This master's thesis project is focused on the study of the *Tricholoma equestre* mushroom species, which has not been much studied until now. The scope of the work was to investigate the effect of polysaccharides and of phenolic compounds, extracted from *Tricholoma equestre* species, on the production of ROS in neuronal cells. The experiments were performed in brain slices from *Wistar* rats, at the mossy fiber synapses from CA3 hippocampal area. Polysaccharides and phenolic compounds extracts obtained from the *Tricholoma* mushroom, were used to prepare different solutions with concentrations of 0.1, 0.5 and 1 $g.L^{-1}$, by adding the extracts to the artificial cerebrospinal fluid (ACSF), which has the composition of the neuronal extracellular medium. The aim of this part of the work was to study the effect of those compounds on neuronal ROS production using the fluorescent ROS indicator H_2DCFDA.

The results show that the lower polysaccharides concentrations (0.1 and 0.5 $g.L^{-1}$) did not cause significant ROS changes while the higher dose (1 $g.L^{-1}$) led to a decrease of the ROS signals of about 2.7 % with respect to the baseline. The experiments carried out with the phenolic compounds solutions gave more accentuated changes that were opposite to the previous one. The larger increases in ROS production were observed for the concentrations of 0.1 and 1 $g.L^{-1}$, having the fluorescence signals amplitudes of about 18 % and 14 %, respectively, while for 0.5 $g.L^{-1}$ the enhancement was approximately 6 %. These facts indicate that the phenolic compounds have a peculiar concentration dependent effect, occurring the

larger ROS changes for the lower concentration, 0.1 g. L^{-1}, whose signals have a similar profile to that of the higher concentration, 1 g. L^{-1}.

The last group of experiments was made with the synthetic compound, 4-hydroxybenzoic acid (1 g.L^{-1}), which is the only identified phenolic compound of the studied mushroom species. The results indicate that this acid causes similar changes to those observed for the same concentration of the phenolics extract, with a maximum value of about 14 % upon the removal of the acid.

In conclusion, and due to the obtained results, it is undeniable that mushrooms have antioxidant properties that affect the performance of cellular metabolism, acting on ROS signals and triggering different responses of the organism to these stimuli.

KEYWORDS: Hippocampal CA3 area; H_2DCFDA; phenolic compounds; polysaccharides.

Resumo

Atualmente, é evidente a rápida taxa de crescimento da população afetada com envelhecimento prematuro e por numerosas doenças como o cancro e doenças neurodegenerativas, tais como as doenças de Alzheimer, de Parkinson e a esclerose lateral amiotrópica, que são as mais frequentes e que requerem mais atenção. Uma das causas principais para o início destas doenças é a produção descontrolada de espécies reativas de oxigénio (ROS), derivadas da produção excessiva de radicais livres. De forma a reduzir danos oxidativos e considerando a restrição no uso de antioxidantes sintéticos, verifica-se uma procura crescente de produtos naturais com um elevado potencial antioxidante.

Entre os produtos naturais existentes os cogumelos têm sido objeto de inúmeros estudos devido aos seus efeitos benéficos na saúde para além das suas propriedades nutricionais. Estes são fungos muito ricos em compostos bioativos, tais como polissacarídeos, compostos fenólicos, vitaminas e metabolitos secundários. Alguns dos compostos referidos tem muitas propriedades antioxidantes que são muito benéficas para o organismo. Este projeto de tese de mestrado foca-se no estudo da espécie de cogumelos *Tricholoma equestre* que, até à data, não tem sido muito estudado. O objetivo do estudo era investigar o efeito de polissacarídeos e de compostos fenólicos, extraídos da espécie *Tricholoma equestre*, na produção de ROS em células neuronais. As experiências foram realizadas em fatias cerebrais de ratos *Wistar* nas sinapses das fibras musgosas da área CA3 do hipocampo. Extratos de polisacarídeos e de compostos fenólicos, obtidos do cogumelo *Tricholoma*, foram usados para preparar diferentes concentrações de 0.1, 0.5 e 1 $g.L^{-1}$, adicionando os extratos ao fluído cerebroespinal artificial (ACSF), que tem a composição do meio neuronal extracelular. O objetivo desta parte do trabalho era estudar o efeito daqueles compostos na produção de ROS neuronal usando o indicador de ROS fluorescente H_2DCFDA.

Os resultados mostram que as concentrações mais baixa de polissacarídeos (0.1 and 0.5 $g.L^{-1}$) não causaram variações significativas de ROS enquanto que a dose mais alta (1 $g.L^{-1}$) levou a uma diminuição dos sinais de ROS de cerca de 2.7 % em relação à linha base. As experiências realizadas com as soluções de compostos fenólicos originaram variações mais acentuadas que eram opostas à anterior. Os maiores aumentos na produção de ROS foram observados para as concentrações de 0.1 e 1 $g.L^{-1}$, tendo os sinais de fluorescência respetivamente amplitudes de cerca de 18 % e 14 %, enquanto que para 0.5 $g.L^{-1}$ o aumento era

aproximadamente 6 %. Estes factos indicam que os compostos fenólicos têm um efeito dependente da concentração que é peculiar, ocorrendo as maiores variações de ROS para a menor concentração, 0.1 $g.L^{-1}$, cujos sinais têm um perfil semelhante aos da concentração mais elevada, 1 $g. L^{-1}$.

O último grupo de experiências foi feito com o composto sintético 4-hydroxybenzoic acid (1 $g.L^{-1}$), que é o único composto fenólico identificado na espécie de cogumelos estudada. Os resultados indicam que este ácido causa variações de ROS semelhantes às que foram observadas para a mesma concentração do extrato de fenólicos, com um valor máximo de cerca de 14 % após a retirada do ácido.

Em conclusão, e pelos resultados obtidos, é inegável que os cogumelos possuem propriedades antioxidantes que afetam o desempenho de metabolismo celular, atuando nos sinais de ROS e desencadeando diferentes respostas do organismo a estes estímulos.

PALAVRAS-CHAVE: Área CA3 do hipocampo; H_2DCFDA; compostos fenólicos; polissacarídeos.

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List of abbreviations and Acronyms

- **AMPA:** Amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
- **ASCF :** Artificial Cerebrospinal Fluid
- **ATP:** Adenosine Triphosphate
- **BA:** Benzoic Acids
- **BHA:** Butylated Hudroxyanisole
- **BHT**: Butylated Hydroxytoluene
- **CA:** *cornu ammonis*

- **CA1:** *cornu ammonis 1*
- **CA2:** *cornu ammonis 2*
- **CA3:** *cornu ammonis 3*
- **CNS:** Central Nervous System
- **DAD:** Diode Array
- **DMSO**: Dimethyl sulfoxide
- **EQ:** Ethoxyquin
- **GABA:** Gama Amino Butyric Acid
- **H_2DCFDA:** 2',7'-dichlorodihydrofluorescein diacetate
- **HPLC:** High Performance Liquid Chromatography
- **HO^-:** Hydroxyl
- **NO^-:** Nitric Oxide
- **NMDA:** N-methyl-D-aspartate
- **$O2^-$:** Superoxide
- **RNA:** Ribonucleic Acid
- **RO^-:** Alkoxyl
- **ROO^-:** Peroxyl
- **ROS:** Reactive Oxygen Species
- **Se:** Selenium
- **TBHQ:** Tert-butylhydroquinone
- **TEAC:** Trolox Equivalent Antioxidant Capacity
- **XRF:** X-ray Fluorescence Spectrometry
- **Zn:** Zinc

1. Introduction

In this chapter will be approached the main theoretical basis underlying this work. A brief description will initially be made about hippocampal anatomy, synapses, reactive oxygen species (ROS) and its respective indicator of ROS. Posteriorly will be given some context about antioxidants compounds and their effects in human health, as well as detailed insight about one particular specie of mushroom (*Tricholoma equestre*), whose effect on human body will be the scope of this work.

1.1. Hippocampal Neuroanatomy

Since many years it has been known that the human brain is constituted by millions of neurons. Each part of the brain is the beginning of other endless systems, with a wide range of brain areas organized into a network absolutely different from the remaining nervous system.

One of the most important areas of the central nervous system is the hippocampal formation, wich is consists of a set of brain parts, constituted by the dentate gyrus (*fascia dentata*), hippocampus, subiculum, presubiculum, parasubiculum and entorhinal cortex (Andersen, Morris, Amaral, Bliss, & O`Keefe, 2007).

The constituents of the hippocampal formation are the same in all mammals, making this area one of the most interesting in neuroscience studies. Thus, there are many scientific studies of the hippocampus, by scientists and clinicians, whose work is intended to contribute for the development of new therapies, for instance, concerning epilepsy and Alzheimer's disease (Andersen et al., 2007).

One of the most important characteristics of the hippocampus is its neuroanatomy, which will be briefly described next. The hippocampal formation has a "C" form and three subdivisions in the CA *(cornu ammonis)* area: CA1, CA2 and CA3. These subareas were identificated by the neuroanatomist Lorente de Nó and his teacher Ramon y Cajal (Andersen et al., 2007). Figure 1 shows the hippocampal formation with the referred areas and the main neuronal circuits.

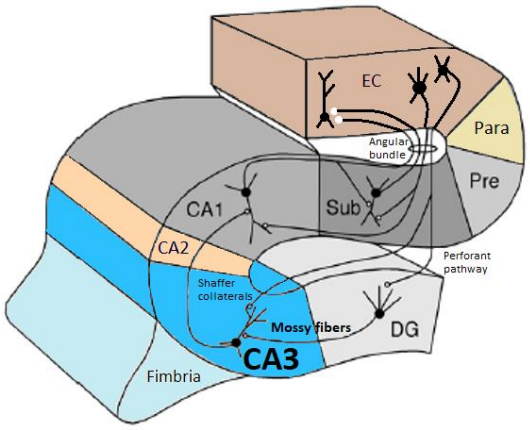


Figure 1: Hippocampal formation with the different structures.

The hippocampus is composed of two main types of cells, which act together: pyramidal cells of *cornu ammonis* (areas CA1, CA2 and CA3) and granule cells of the dentate gyrus (Amaral & Witter, 1989). Area CA1 comprises pyramidal cells and is responsible for matching and mismatching the information that comes from CA3 area. Area CA2 isn`t considered a very important hippocampal subdivision except in cases of epileptic damage(Andersen et al., 2007).

The pyramidal cells of CA2 and CA3 areas contain a lot of projections to all parts of the hippocampus and in some parts of CA3 area are very similar to the branches of the dentate gyrus. The CA3 area is located near the dentate gyrus and tends to project to the distal portion of CA1, near the subiculum(Anand & Dhikav, 2012). The pyramidal neurons in CA3 and CA1 zones are structurally alike.

The CA3 area has been highly studied because of the mossy fibers projection from the granule cells of dentate gyrus, due to their unique functional specializations and because of the formation of the high length axon collaterals between CA3 neurons, which create an interconnection network between them(Andersen et al., 2007).

The mossy fibers were the aim of many studies performed by the classical Golgi anatomists, by Ramon y Cajal and Lorente de Nó (Amaral & Witter, 1989) The first experimental study on this fibrous network was conducted by Blackstad *et al.*(Amaral & Witter, 1989), who visualized the distribution of mossy fibers in the rat hippocampus. They are characterized by large protuberances with synaptic vesicles that contain, besides the excitatory neurotransmitter glutamate, a large concentration of free or chelatable zinc.

Over the last years, hippocampal neuronal cells have been very important on discovering the messages transmitted at synapses, due to continuous changes in neural activity.

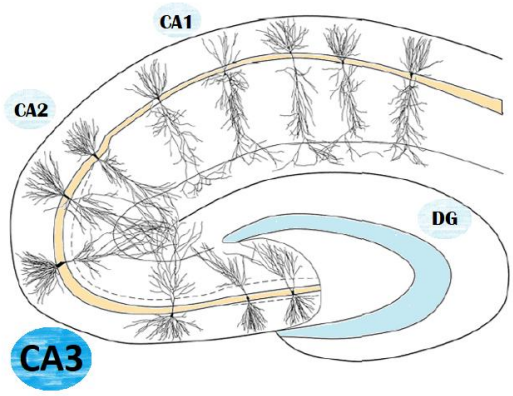


Figure 2: Transverse hippocampal slice.

1.2. Synapses

The human brain contains at least 100 billion neurons, which interact with many other cells. Particularly in the hippocampus there is a lot of information to be communicated to a large number of elements, across sophisticated and highly efficient mechanisms. This communication is only possible through the action of synapses, which enable functional contact between neurons consisting of several steps (Purves et al., 2004). Firstly, the information transmitted through the synapses is provided by an individual neuron. Secondly a one-way valve has the function of making the transmission from the pre- to the postsynaptic neuron. Lastly, synaptic plasticity, i. e. the change in the strength of synaptic responses due often to intense stimulation, may underlie memory formation and storage in cells (Purves et al., 2004) (Pereda, 2014).

Initially physiologists believed that signal transmission between neurons was made, only and exclusively, by physical connection. However, the studies performed by Golgi, Ramon y Cajal and others discovered the existence of the other kind of synapses, in which the pre- and postsynaptic neurons were close to each other but not continuous(Amaral & Witter, 1989). Oliver and Shäfer, Langley and Elliot have shown that neuronal transmission could have a chemical nature. So, although synapses are presented in different forms and shapes and have distinct properties, they can be distinguished, according to their mechanisms of transmission, in two types: electrical and chemical synapses (Holz & Fisher, 1999).

Electrical synapses are found in all nervous systems allowing the passage of electrical current from one neuron to another. The current flow, which occurs through intercellular channels, allows the transfer of ions and small molecules from the presynaptic to the postsynaptic membrane. These two membranes are linked together by an intercellular specialization called a gap junction (Pereda, 2014).

Besides the transmission of ions during the electrical activity, gap junctions also allow the transference of various small molecules, such as cAMP or IP3, which may be important for cellular signaling in the brain (Pereda, 2014) (Purves et al., 2004) (Hennig, 2008).

In contrast, at chemical synapses the information is transferred through the secretion of neurotransmitters, existing no direct flow of current from the pre- to the postsynaptic neuron (Figure 3). The neurotransmitters are chemical agents, stored in presynaptic vesicles, which upon release from the presynaptic membrane may act on specific receptors located at the postsynaptic membrane(Purves et al., 2004) (Pereda, 2014).

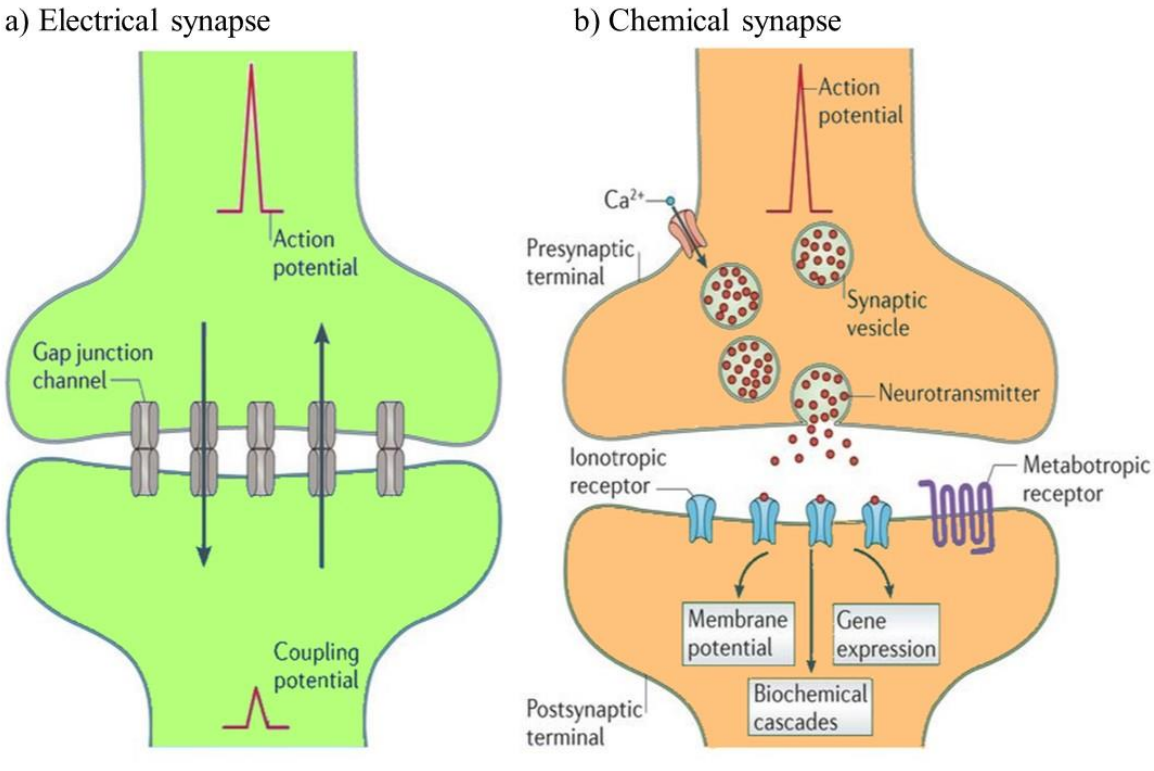


Figure 3: Synaptic transmission. a) Electrical synapse and b) Chemical synapse (Adapted from Electrical synapses and their functional interactions with chemical synapses(Pereda, 2014)).

X'Within the chemical synapses there are two types of signals: excitatory and inhibitory. These depend on the neurotransmitter released and how they affect the postsynaptic neuron. The possibility of occurring a response at the postsynaptic region, leading to the formation of a

nervous impulse depends on the type and amount of neurotransmitter reaching the synaptic cleft, which in turn, results from the presynaptic stimulation. If the membrane potential in the postsynaptic neuron increases, this leads to an excitatory postsynaptic potential. Otherwise, a postsynaptic inhibitory response is formed (Purves et al., 2004).

The most important transmitter in the hippocampus, in the mammalian central nervous system (CNS), is glutamate and the major inhibitory transmitter is GABA (gama amino butyric acid), which is synthetized by decarboxylation of glutamate (Andersen et al., 2007) (Purves et al., 2004).

There are many types of glutamate receptors and three of them are ionotropic: NMDA (*N*-methyl-D-aspartate), AMPA (-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) and kainite receptors. These have an ion channel activated by glutamate binding to specific receptor sites, causing excitatory postsynaptic responses (Andersen et al., 2007) (Purves et al., 2004).

The ionotropic glutamate receptors are non-selective and when activated by neurotransmitters allow the passage of Na^+ and K^+ ions and, in some cases, also of Ca^+ ions (Purves et al., 2004) (Andersen et al., 2007).

1.3. Reactive Oxygen species (ROS)

Reactive oxygen species (ROS) serve as signaling molecules to regulate biological and physiological processes. They are derived from oxygen and can oxidize other molecules so that they are considered as toxic byproducts of aerobic metabolism and can be used to describe many different molecules and free radicals derived from molecular oxygen (Schieber & Chandel, 2014) (Sena & Chandel, 2012) (Turrens, 2003).

Mitochondria are one of the most important sources of ROS within eukaryotic aerobic organisms. It's metabolic work cannot be performed without oxygen which is involved in oxidative reactions in order to satisfy energy requirements. However, as oxygen can behave as a toxic agent, excess of ROS is often associated with the principle of oxidative stress which can affect mitochondrial metabolism therefore inducing cellular damage and physiological dysfunction such as in lipids, proteins and nucleic acids, being the final outcome neurodegenerative diseases, diabetes, cancer, and premature aging(Murphy, 2009) (Sena & Chandel, 2012) (Venditti, Di Stefano, & Di Meo, 2013) (Zorov, Juhaszova & Sollott, 2014) (Schieber & Chandel, 2014).

The most abundant ROS in a living cell can present itself in the form of radicals such as the

superoxide anion (O_2^-) and hydroxyl radicals (•OH) and as non-radicals like hydrogen peroxide (H_2O_2) and singlet oxygen $^1O_2$ (Zorov, Juhaszova & Sollott, 2014) (Schieber & Chandel, 2014)

The oxygen consumed by mammals is reduced to water in a reaction catalyzed by mitochondrial cytochrome oxidase. However, mitochondrial electron transport chain contains many redox centers that are able to transfer an electron to oxygen, generating the $O_2^{\bullet -}$. The enzymatic dismutation of this radical yields H_2O_2, which, in turn, can produce the highly reactive •OH (Venditti et al., 2013).

ROS appear to be involved in the regulation of various channels and receptors, such as NMDA receptors, potential-dependent calcium and potassium channels, and also in synaptic plasticity (Massaad & Klann, 2011). Although the role of the mitochondrial ROS is not yet fully understood, it is known that excessive ROS production caused by mitochondrial dysfunction may be responsible of several diseases (Ames, Shigenaga, & Hagen, 1993). Mitochondrial ROS can also act as signaling molecules to activate pro-growth responses (Sullivan & Chandel, 2010).

When talking about Ca^{2+}, it is important to note that it promotes synthesis of ATP by stimulating enzymes of the Krebs cycle and oxidative phosphorylation in the mitochondria. An increased metabolic rate consumes more oxygen and increases respiratory chain electron leakage and ROS levels (Brookes, 2004). Under normal conditions, Ca^{2+} typically diminishes ROS from mitochondrial complexes I and III while ROS generation is enhanced when these complexes are inhibited by some kinds of external agents.

The metabolic state of the mitochondria determines the effects of calcium on mitochondrial ROS levels. When the membrane potential is high (no ATP synthesis), Ca^{2+} uptake results in decreased ROS generation. When the membrane potential is depolarized (ATP synthesis), ROS generation is normally stimulated (Adam-Vizi & Starkov, 2010). When mitochondria are overloaded with Ca^{2+}, ROS production might increase independently of the metabolic state of mitochondria (Görlach, Bertram, Hudecova, & Krizanova, 2015).

Natural systems can play an important role when protecting against ROS overproduction (redox buffering systems such as reduced glutathione, vitamins, albumins and globulins, free fatty acids, etc) and are considered as an essential source of antioxidants.(Zorov et al., 2014).

1.4. Fluorescent ROS indicator

Testing oxidative activity in living cells can be a true challenge when talking about the presence of several reactive oxygen species in one same cell. (Kalyanaraman et al., 2012)

Variations of ROS by the addition of phenolic compounds or polysaccharides were measured using the 2',7'-dichlorodihydrofluorescein diacetate indicator, H_2DCFDA. The chemical formula of this organic compound is $C_{24}H_{16}Cl_2O_7$, which can be represented as seen in Figure 4 (ThermoFisher Scientific, 2010)

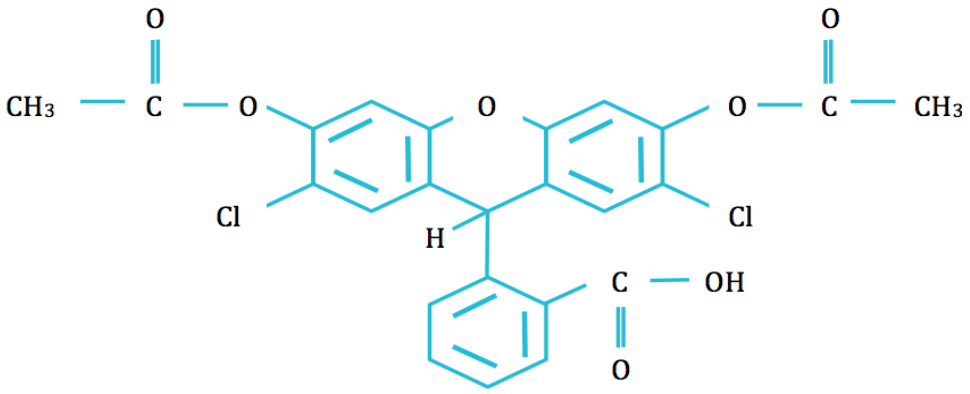


Figure 4: Chemical structure of 2',7'-dichlorodihydrofluorescein diacetate indicator. (Adapted from ThermoFisher Scientific, 2010).

This fluorogenic dye, is used, among other applications, to detect oxidative stress within a cell. It is usually used to measure hydroxyl, peroxyl and other reactive oxygen species (ROS). H2DCFDA is diffused into the cell and deacetylated by cellular esterases forming a non-fluorescent compound which is consequently oxidized by ROS into DCF, 2', 7' – dichlorofluorescein. This is a highly fluorescent compound that can be monitored by many fluorescence-based techniques like confocal microscopy, flow cytometry however it is usualy detected by fluorescence spectroscopy with an excitation and emission spectra of, approximately, 495 nm and 529 nm respectively (ThermoFisher Scientific, 2010) (ABCAM, 2018) (Kalyanaraman et al., 2012).

1.5. Antioxidants - Phenolic and Polysaccharides compounds

With the increasing number of diseases caused by oxidative damage, due to the action of free radicals in cell metabolism, it is very important for humans the search for new natural products, with antioxidant properties. The same is true for antioxidant supplements and antioxidant food, which may be used to reduce the oxidative negative cellular effects. This happens when the electron flow is decoupled and free radicals are generated. These are known

as reactive oxygen species (ROS) and include superoxide (O_2^-), hydroxyl (HO^-), peroxyl (ROO^-), alkoxyl (RO^-) and nitric oxide (NO^-) (Gülçin, 2012).

On the other hand, oxidation is important in the production of energy that is responsible for aerobic life and its metabolism, in biological process. That is, oxidation is defined as the transfer of electrons from one atom to another, being oxygen the final acceptor in a flow of electrons, in a process that leads to the production of energy in the form of adenosine triphosphate (ATP).

The production of free radicals derived from ROS is responsible for the appearance of many diseases, such as metabolic diseases, heart diseases, some cancers, rheumatoid arthritis, cirrhosis, atherosclerosis, severe neural disorders such as Alzheimer´s and Parkinson`s, and also of the degeneration of tissues that cause premature aging (Kozarski et al., 2015) (Ribeiro et al., 2006) .

The antioxidant market is divided in two different types: natural and synthetic antioxidants. Natural antioxidants are considered to be compounds such as plant and fungal extracts (like mushrooms), spices (rosemary, thyme, marjoram, oregano, sage, basil, pepper, clove, cinnamon and nutmeg), flavonoids, ubiquinol (fully reduced form of coenzyme Q10), glutathione, zink (Zn), selenium (Se), vitamin A (including carotenoids), vitamin C and vitamin E (including tocopherols and tocotrienols). The following compounds are considered synthetic phenolic antioxidants: butylated hudroxyanisole (BHA), butylated hydroxytoluene (BHT) and others such as propyl gallate, tert-butylhydroquinone (TBHQ) and ethoxyquin (EQ), all of which inhibit oxidation.

However, in the last few years there have been major restrictions on the use of synthetic antioxidants, mainly BHA and BHT, thus causing a growing gradual interest in natural antioxidants and raising the interest for example for mushrooms. (Kozarsky, 2015).
Mushrooms are rich in bioactive compounds, such as vitamins, polysaccharides and polyphenols and secondary metabolites in their fruit bodies and some of them are full of antioxidant properties, having very beneficial effects in the human body (Robaszkiewicz, Bartosz, Ławrynowicz, & Soszyński, 2010).

Polyphenols are the most commonly found antioxidant compounds in ingested foods. They have a high number of phenolic compounds, and so it is sometimes difficult to understand

the many biological activities that occur and their effects on human health. Polyphenols may be present in food in the form of esters, glycosides or polymers, but these substances are not ingested in this form, being hydrolysed by intestinal enzymes (Kozarski et al., 2015).

In addition to their antioxidant properties polyphenols have also shown a pro-oxidant capacity, characterized by the formation of reactive oxygen species or by the inhibition of antioxidant compounds(Kozarski et al., 2015). Pro-oxidants may thus affect the cellular redox potential and generate two effects: decreased levels of oxidized proteins and lipids or increased levels of oxidant scavenging proteins(Kozarski et al., 2015).

Furthermore, the polyphenols have the capacity to interact with steroid receptors, leading to changes in the mitochondrial transmembrane potential and, consequently, to changes in ROS activity, which depend on the cellular system. Therefore, polyphenols can enhance cell survival by acting as antioxidants and also slow down diseases, such as tumor growth, through higher ROS formation, causing oxidative damage due to their pro-oxidant ability(Wei, Helsper, Leonardus, Lambertus, & Van, 2008)(Kozarski et al., 2015).

1.5.1. Mushroom specie Tricholoma equestre

Etymologically, the generic name Tricholoma derives from ancient Greek *trichos* (τριχος), meaning *hair* and *loma* (λωμα), meaning *fringe* or *border*(Sven & Persson, 1977).

Formerly, mushroom taxonomy considered a variety of mushrooms based on their physical appearance. Now, the advances in mushroom taxonomy show that following a more complete scientific analysis, new groups emerge(Kuo, 2006).

Some species are easy to identify, especially because of the ring, the texture of the hat and the color, but in some cases the determinant characteristics are the odor and the taste. However, there is a group of species that have grey and brown colorations, which because of their morphological similarity are difficult to distinguish.(Kuo, 2006).

The mushroom species *Tricholoma*, in general, originated in North America. These mushrooms, usually referred to as cavaliers or knights, are relatively robust and have a fleshy stipe and blades, being some quite colorful. They ave fibrous fruitibodies and, when wet, may be tacky or viscid and the lamellae are white or shaded with colours similar to those of the cap

or stem(Kibby, 2010). This species belongs to the large genus *Agaricus,* which includes all of the gilled mushrooms(Kibby, 2010) (Oyetayo & Ariyo, 2013)(Oyetayo & Ariyo, 2013), and to phylum Basiodyomicota and included includes a set of fungi mycorrhizal, which are easyly recognized in the vegetation due to their unique morphological properties.

The species grows on the ground, in the brink of the deep seated of trees, in late summer or even in spring, in places of warmer temperature. Most Tricholomas are good edibles, but some are inedible or even poisonous. Their is not very wellknown yet, so anyone should be careful when eating mushrooms of this species and always make their identification before. (Biblio: Tricholomas of North America (Bessette *et all*, 2012).

Kumm defines Tricholoma equestre as the current mushroom name being his synonimous defined as Tricholoma flavovirens (Lundell) and *as* Tricholoma auratum (Gillet)(Mycobank)(Silva *et al,* 2013).

In addition to the scientific name, Tricholoma equestre is popularly called by “yellows”, “míscaro”, “yellow míscaro” or “tortulho” (Figure 5).

Figure 5: Mushroom specie Tricholoma equestre (Silva *et al*., 2013)

The species of *yellow Tricholoma* are restricted to Scottish pinewoods and have some records in England an in Europ. (kibby). The Taxonomic Hierarchy of *Tricholoma equestre* fungi and the main characteristics of this species are presented in Table 1 and Table 2, respectivelly.

Table 1 - Taxonomic Hierarchy of *Tricholoma Equestre* Mushroom (Mushroomexpert, 2018) (

Taxonomic Hierarchy	Tricholoma equestre
Phylum	Basidiomycota
Subphyllum	Agaricomycotina
Class	Agaricomycetes
Order	Agaricales
Family	Tricholomataceae
Genera treated	*Tricholoma*

Table 2 - Characteristics of Tricholoma Equestre (Mushroomexpert, 2018) (Isabel, 2011).(Silva et al., 2013).

Characteristics	Tricholoma equestre
Morphologycal Characteristics	
Cap	
∅ (cm)	3-12
Form	Young: Conical to broadly convex and the margin initially rolled under somewhat. Adult: nearly flat and slightly mamelonade. Finaly usually depressed, curve margin, after right and sometimes recurved.
Coating/cuticle	Sticky when fresh, but separable, thick, viscous, bright when dry.
Color	Bright yellow or yellow brass in the margin when young and fresh. Progressively reddish-brown with some nuances of bronze in the center or often with an olive brown or brownish center. Becomes yellow-brown by maturity;
Gills	Attached to the stem, often by means of a notch, with pale to bright yellow color.
Blades	Smooth or with a few appressed fibers over the center, with a yellow sulfer color or sometimes citrine. Fragile and unequal.
Steam	
Long (cm)	2-10
Thick (cm)	Up to 2

Form	Sub-equal or with an enlarged base; smooth or very finely hairy.
Color	Cylindrical, sometimes slightly broad at the base, massive, with smooth fibrillous surface, yellow in color equal to that of the blades or lighter.
Flesh	
Color	White to very pale yellow near the cap surface, yellowish ocher or brass on the periphery. Not changing on exposure.
Odor	Sweet and delightful fungal.
Taste	Lightly floury ou slightly aromatic, mild tasty, remenbering hazelnuts
Other Characteristics	
Chemical Reactions	Ammonia (NH_3) or bases to bright orange (in the flesh). Hidróxido de potássio (KOH) on cap surface reddish (usually when ssociated with spruce)
Microscopic features	
Spores	5-8.5 x 3-6 µm. Smooth, elliptical to subamigdaliformes and inamyloid.
Cystidia	Absent
Clamp connections	Absent.
Marginal hair	Insignificant or tortuous
Hyphae of pileipellis	3-5 µm. More or less bifurcated or interlaced, more fasciculated in the outside, with rare gelled plates on the disc.
Pigment	Mixed to more or less dotted
Subcutis and hypodermis	Progressively thick with no tendency pseudoparenchymal.
Ecology/Habitat	Mycorrizal with conifers, specially pines (species of Pinus). It is also documented with spruces and firs, but its less common. Occasionally growth with madrone and with quaking aspen, but both growths near of conifers. Growing dispersed or aggregated. Exist in abundance in North America and in the fall and winter seasons.
Edibility	Very good. Very appreciated and consumed, despite the warning that it may lead to poisoning. Its marketing is banned in many countries of the European Union.

2. Objectives

This master`s thesis project focused in on the study of the *Tricholoma equestre* mushroom specie. The main objective of this work was the investigation of the effect of

polysaccharides and phenolic compounds extracted from *Tricholoma equestre* species on the production of ROS, in neuronal cells. In addition to these compounds, 4-hydroxybenzoic acid was also studies, being a phenolic compound isolated and abundant in considerable quantities in the study mushroom specie.

Polysaccharides and phenolic compounds extracts were prepared with ACSF in solution of 0.1, 0.5 and 1 g. L^{-1} and the 4-hydroxybenzoic acid in concentration of 1 g. L^{-1}. After, these solutions were introduced in brain slices from Wister rats, at the mossy fibers from CA3 hippocampal area.

In this way, we intended to study the effect of those compounds on the production of neuronal ROS, using the fluorescent ROS indicator H_2DCFDA.

3. State of the art

3.1. The Hippocampus

The hippocampus is one of the most studied parts of the brain and has many functions, including in human learning and memory formation as well as in feeling and reacting. It is involved in two kinds of memory, declarative memory (relates facts and events) and spatial memory (involving pathways and routes). If the hippocampus is affected by illness or adverse conditions, a loss of memory may occur. The major diseases that affect the hippocampal functions are Alzheimer´s disease, epilepsy, depression and stress(Kozarski et al., 2015).

3.2. Mushrooms

The mushrooms continue to be the subject of many studies in the health and nutrition sciences. In particular, the mushroom species *Tricholoma equestre* is the focus of this master thesis work.

3.2.1. Mushrooms and health

The percentage of people currently suffering from cancer and neurodegenerative diseases, such as amyotrophic lateral sclerosis, Alzheimer's, Huntington's and Parkinson's has been increasing. Since the currently available therapies are not yet fully effective, there is a search for alternatives to mitigate their effects. The appearance of these diseases is directly related to the uncontrolled production of oxygen, derived from the excessive production of free radicals(Ribeiro *et al*., 2006).

For these reasons, strategies have been adopted at the level of neuroprotection, trying to prevent damage to the neuronal structure or even death (Lemieszek, Nunes, Cardoso, Marques, & Rzeski, 2018)**.**

Some phytochemicals, polysaccharides, phenolic compounds and organic acids are involved in the protection of the human body of many diseases, due to their antioxidant potential and also for maintaining fruit and vegetables quality and organoleptic characteristics*(Ribeiro et al.*, 2006)**.**

The polysaccharides are classified as the class that has the largest constituents of edible and medicinal mushrooms, being considered as adaptogen, immunostimulators and antioxidants. This antioxidative property is justified by its scavenging ability, its reduction property and the ability to form Fe^{+} chelators. They are also involved in lipid peroxidation, erythrocyte hemolysis and in increased enzymatic activities in eukaryotic and prokaryotic cells,

as well as in acting in antioxidant processes, such as in the enzymes SOD, CAT and GPx (S. P. Wasser & Weis, 1999) (S. Wasser, 2014).

Polysaccharides that are found on the cell wall of fungi can be connected by covalent bonds with proteins, resulting from lignin degradation processes. On the contrary, there is a greater antioxidant capacity for polysaccharide fractions in their pure state. For example, the extraction of *Agaricus brasiliensis* polysaccharides mushrooms obtained by deproteinization pronase, demonstrated a high antioxidant activity against •OH and $\bullet O_2^-$ radicals (Kozarski *et al.*, 2014)

Besides the mitochondria and other intracellular organelles ROS are also produced within the gastrointestinal tract (GI), and despite the protective barrier that the organism possesses, the pathogenic agents can lead to oxidative lesions in the GI, attacking the immune cells. The high antioxidant capacity of polysaccharides from edible mushroom species can prevent lipid peroxidation and the appearance of several gastrointestinal diseases, such as peptic ulcers, gastrointestinal cancers and intestinal inflammation due to oxidative stress (Kozarski *et al.*, 2015).

Until now no cytotoxic effect of fungal polysaccharides has been reported on normal cells. On the contrary, it was found that the application of a *Ganoderma lucidum* polysaccharide extract led to the proliferation, *in vitro*, of trophoblast cells, which are an essential factor for fetal growth in humans (Batbayar, Lee, & Kim, 2012).

Phenolic compounds are the secondary metabolites in plants, all with a common aromatic ring and one or more hydroxyl groups. It has been found that, to date, there are about 8000 natural phenolic compounds. Most of them are isolated from plant sources, such as simple phenols, flavonoids, lignins and lignans, tannins, xanthones, and coumarin. Some studies report how phenolic compounds act beneficially at the level of cancer treatment (Anantharaju, Gowda, Vimalambike, & Madhunapantula, 2016).

In a recent study the pro-oxidative capacity of polyphenols was confirmed due to the fact that they act as photosensitizers in the generation of O2 (Lagunes & Trigos, 2015).

Given that O2 is a powerful oxidizing agent and causes oxidation in biological systems, it was confirmed in this study that the two polyphenols studied (resveratrol and curcumin) favored a pro-oxidant effect against normal cells (Kozarski et al., 2015). Thus, it is also likely that similar processes occur with mushroom polyphenols, requiring special attention to possible

harmful redox effects of mushrooms (Bilski, Li, Ehrenshaft, Daub, & Chignell, 2000) (Lagunes & Trigos, 2015) (Das & Das, 2002).

Since the species studied in this work is *Tricholoma equestre* the issue of being or not an edible mushroom is addressed next..

Most of the times it is difficult to distinguish edible *Tricholoma* mushrooms from those which are suspected to be toxic or are really poisonous and deadly above certain doses. However, it has been shown that no *Tricholoma* is dangerously poisonous in small amounts. The existing information about poisoning effects indicates that they were only toxic when they were consumed in large amounts.(Bessette et al., 2012).

There are no specific scientific studies on the safety of the "Yellow Knight" mushroom *Tricholoma equestre* (L.P.Kumm). However, one of the studies reported twelve cases of poisoning, including three fatalities, in France, due to Tricholoma equestre mushrooms consumption (Bessette et al., 2012). All fatalities and the remaining affected people consumed large amounts of *Tricholoma equestre* in consecutive meals. All affected people presented fatigue and muscle weakness accompanied by myalgia, especially in the upper legs, 24 to 72 hours after the last meal where they consumed the mushrooms. In a period of 3 to 4 days, they became worst, with increasing weakness and the appearance of other symptoms such as stiffness in the legs, nausea, vomiting and deep sweating. This symptomatology led physicians to diagnose rhabdomyolysis in patients. Rhabdomyolysis is a pathology that affects and destroys muscle tissues. According to medical reports no patient had a history of trauma or of the use of medication that might explain the occurrence of rhabdomyolysis (Bedry *et al*, 2001)

Besides this fatality in France, similar poisonings have also been reported in Poland. However, in North America, this mushroom species has been used in food, for many years, without any health problems (Bessette et al., 2012).

Data based on national legislation and information from mycologists report the status of edibility of *Tricholoma equestre* in European countries. *Tricholoma equeste* is then considered poisonous in some countries, due to the rare cases of rhabdomyolysis and the possibility of being a toxic mushroom. (Bedry & Gromb, 2009). While in others it is considered an edible mushroom, such as in some parts of Asia, Europe and North America, where it is caught and consumed by many people every year(Rzymski & Klimaszyk, 2018). Nevertheless, in some of these countries people are warned that it may cause some type of poisoning, depending on the doses consumed.

Based on the existing information, it can be considered that *T. equestre* should not be considered a toxic species because it presents no more health threat than other species of mushrooms currently available in nature. In addition, further study should be made of reported cases of poisoning by this species, by analyzing other factors that may have interfered with and caused the poisoning (Rzymski & Klimaszyk, 2018).

3.2.2. Mushroom Antioxidant Compounds: Polysaccharides, Polyphenols and Organic Acids

The discovery that mushrooms have neuroprotective and anticancer properties led to several studies including one with the mushroom species *Cantharellus cibarius* (the golden *Chantarellus*), because it is a cosmopolitan species, widely consumed and appreciated for its smell and taste (Lemieszek et al., 2018). Futhermore, recent reports show that this species is already known for its beneficial effects in terms of anticancer, anti-inflammatory, immunomodulatory, antigenotoxic, antiaging and antioxidant properties. The aim of these studies was the evaluation of neuroprotective properties of polysaccharide fractions isolated from *C. cibarius*, in various models of in vitro neurodegeneration, including throphic stress, excitotoxicity and oxidative stress. These studies were performed in neurons from the human neuroblastoma cell line SHSY5Y (Lemieszek et al., 2018).

The studies carried out with *C. cibarius* polysaccharide extracts addressed effects on neuronal cells by the use of Neurite Outgrowth Staining, MTT and LDH tests. In addition, their antioxidant capacity was measured through commercial antioxidant tests, using the ABTS technique and the fluorescent indicator 2 ', 7'-dichlorofluorescein (DCFDA) for cellular ROS detection. In relation to the ABTS technique, the results presented are based on the Trolox Equivalent Antioxidant Capacity (TEAC) method, which measures the concentration of the substances exhibiting antioxidant capacity corresponding to 1 mM Trolox. (Lemieszek et al., 2018).

The conclusions revealed that the fractions of *C. cibarius* (CC2a, CC3) has positive effects on neuronal viability and neurite outgrowth, both under normal and under stress conditions. For all the studied fractions, there were positive effects on antioxidant capacity and also on the ability to neutralize the changes induced by glutamate receptor agonists (glutamate, NMDA and AMPA) (Lemieszek et al., 2018).

This study concluded that the compounds under study have great potential in the development of effective therapeutic strategies for neurodegenerative diseases. However, despite these data there is still a lack of information on the direct impact of *C. cibarius* and its compounds in the central nervous system. This study was the first to evaluate the neuroprotective activity of the polysaccharide fraction of *C. cibarius* in neurodegeneration (Lemieszek et al., 2018).

A recent study about the identification of certain mushroom compounds, was performed by Pessoa (2017). The main objectives were the characterization of four species of mushrooms: *Boletos edulis, Tricholoma equestre, Ganoderma Lucidum* and *Ganoderma Lingzi*, based on the studies described next. The first group of experiments were based on the extraction of two types of antioxidants, polysaccharides and phenolic compounds, and on the analysis of the total phenolic content, using the method of Folin-Ciocalteu. The type of phenolic compounds in each mushroom was also determined through the High Performance Liquid Chromatography (HPLC) technique and the monosaccharide content using chromatography. In addition, measurements of ribonucleic acid (RNA) were performed by Spectral Scanning in the UV/Vis region and a qualitative elementary analysis was made through the X-ray fluorescence spectrometry (XRF) technique, which allows identification of metallic components in the mushrooms (Pessoa, 2017).

Furthermore, the effect of polysaccharides from the species *Boletos edulis* on the formation of neuronal reactive oxygen species (ROS) was also evaluated. These studies were carried out in brain slices, at the hippocampal CA3 area, for different concentrations of the polysaccharide extract. The results of this study suggest that, at the concentration of 1 $g.L^{-1}$, the *Boletos edulis* polysaccharides reduce the amount of neuronal ROS at the studied synaptic system (Pessoa, 2017).

The results also showed that the yield obtained in the extraction of the phenolic compounds was higher than 40 % in the case of the *Boletos edulis* and *Tricholoma equestre* species, but for the two *Ganoderma* species the value was quite low, of the order of 10 %. With respect to the phenolic content, the determined mean values for the*Boletos edulis, Ganoderma lingzy* and *Ganoderma lucidum* were about 27 mg GAE/ g of mushroom extract, whereas for the *Tricholoma equestre* was approximately 13 mg GAE/ g of mushroom extract. For the dried mushrooms, the values were all lower but again the larger amount was for *Ganoderma Lucidum, Ganoderma lingzy* and *Boletos,* about 11 mg GAE/ g dry extract, and only about 6 mg GAE/ g dry extract for the *Tricholoma* (Pessoa, 2017).

The HPLC analysis revealed the existence of several common phenolic compounds in the various mushrooms that could not be identified. The polysaccharide intermediate yields in the case of the *Ganoderma* species were 80 % while for the remaining species they were close to 50 %.

In the analyses of the sugars composition, the following monosaccharides were found: rhamnosis, fuccose, glucosamine, galactose, glucose, mannose and ribose, being galactose the most abundant. The total amount of monosaccharides was found to be, in general, very low having *Boletos edulis* the highest amount of sugars (4,6 mg of sugars per 100 g of dried mushroom).

In the XRF analysis it was possible to identify several chemical elements present in the mushrooms in question, 15 elements in total: magnesium, silicon, phosphorus, sulfur, chlorine, potassium, calcium, manganese, iron, nickel, copper, zinc, selenium, bromine and rubidium. It is known that most of these elements are essential for health, and therefore the mushroom is and used for therapeutic purposes (Pessoa, 2017).

The study of the effect of polysaccharides of the mushroom *Boletos edulis* in neuronal cells revealed that the application of the polysaccharides extract (1 $g.L^{-1}$) induced a reduction of the ROS signals, detected with the fluorescent ROS indicator H_2DCFDA. Thus these compounds may attenuate negative effects of ROS (Pessoa, 2017).

Another study referred that the main simple phenols found in nature are benzoic acids (BA) and cinnamic acids (CA), with 6 to 9 carbon skeletons. These have always a carboxyl group attached to the benzene ring and also one or more hydroxyl and methoxyl groups attached. In addition, the cinnamic acids have also a side chain of unsaturated propionic acid attached to the benzene ring.

The benzoic and cinnamic acid derivatives commonly found in plants are: benzoic, cinnamylic acid, p-hydroxybenzoic acid, P-coumaric acid, protocatechuic acid, caffeic acid, gallic acid, vanillic acid, isovanillic acid, syringic acid, ferulic acid, veratric acid, chlorogenic acid (3-Caffeoylquinic acid) and dicaffeoylquinic acid (cynarine).

It has also been found that these phenolic compounds attenuate the effect of various diseases involving oxidative stress. As regards anti-carcinogenic effects they have advantages in terms of inducing cell arrest, inhibitory oncogenic signaling controlling cell proliferation,

angiogenesis and apoptosis, modulate ROS levels, promote tumor suppressor proteins and differentiate normal cells (Anantharaju et al., 2016).

By *in vitro* observations and pre-clinical and epidemiological studies, the involvement of plant phenolic acids in the retardation of cancer growth was also evaluated (Anantharaju et al., 2016).

In Portugal, Trás-os-Montes is recognized for being a region rich in the production of edible mushrooms. Nine species of these type of wild edible mushrooms, collected there, were studied, namely: *Suillus bellini*, *Suillus luteus*, *Suillus granulatus*, *Tricholomopsis rutilans*, *Hygrophorus agathosmus*, *Amanita rubescens*, *Russula cyanoxantha*, *Boletus edulis*, and *Tricholoma equestre.* All these species are already known for their antioxidant properties, due to their composition in organic acids and phenolic compounds. Of all species referred, *Tricholoma equestre* was the mushroom chosen for the present study (Ribeiro et al., 2006).

The main objective of it was to analyze the chemical composition of that mushroom, produced and consumed in Portugal, based on their antioxidant potential, in relation to the presence of organic acids and phenolic compounds. To obtain the desired results, the HPLC-diode array (HPLC-DAD) and HPLC-UV technics were used, and the extracts were evaluated for their antioxidant potential, according to the method of DPPH (Ribeiro et al., 2008).

Analysis performed using the HPLC-DAD technique, revealed that all these mushrooms contain the following five organic acids: oxalic, citric, malic, quinic, and fumaric acids (…). Some samples also contain aconitic, ketoglutaric, ascorbic, succinic and shikimic acids. The quantification of compounds indicated that malic and quinic acid are the major compounds of the species analyzed, usually followed by citric acid. The ketoglutaric, ascorbic, and shikimic acids are the less abundant compounds in mushrooms species (Ribeiro et al., 2006). Focusing in the *Tricholoma equestre* species indicates that the results are very similar to those of *Boletos edulis*. The total sum of the acids has a value of 94 to 99 % g/Kg of dry matter, where the mailic and quinic acids correspond to 64 % of nonaromatic compounds, followed by citric acid, which has 24 % of organic acids, being these the compounds in greater percentage. Oxalic acid is the compound present in the minor amount, with 2 % of nonaromatic acids.

Studies of phenolic compounds have found that the *T. equestre*, *S. luteus*, and *S. granulatus* species contain two secondary metabolits in the methanolic extracts and identified p-hydroxybenzoic acid in the *A. rubescens*, *R. cyanoxantha*, and *T. equestre* species and quercetin in *S. luteus* and *S. granulates* (Ribeiro et al., 2006).

The presence of p-hydroxybenzoic acid may be a useful factor for quality control of *R. cyanoxantha* and *T. equestre* species, because it is detected in all samples of these two species. A comparison between the amount of phenolic and of organic acids in these species indicates that the *R. cyanoxantha* normally has fewer phenolic content (Ribeiro et al., 2006).

The consumption of mushrooms is growing largely due to its preciousness both nutritional and medicinal. It is known that mushrooms have a lot of healthy properties and are poor in calories, thus becoming a food with high nutritional value (Ribeiro et al., 2006).

A group of researchers studied the composition of 11 wild edible mushrooms species: *Tricholomopsis rutilans*, *Hygrophorus agathosmus*, *Amanita rubescens*, *Russula cyanoxantha*, *Boletus edulis*, *Tricholoma equestre*, *Suillus bellini*, *Suillus luteus*, *Suillus granulatus*, *Fistulina hepatica* and *Cantharellus cibarius.* In spite of the fact that the species *S. bellini*, *H. agathosmus*, *R. cyanoxantha*, *T. equestre*, and *F. hepatica* have been widely consumed, there were no previous studies of these type of compounds for these mushrooms (Ribeiro et al., 2008).

To determine the qualitative and quantitative profile, a dabsyl chloride procedure was performed, followed by an HPLC-Vis analysis. Dabsyl chloride is a chromophore labeling reagent that allowed the detection of amino acids in HPLC reverse phase, using visible light (Ribeiro et al., 2008).

In all studied species, the presence of the following 20 amino acids was verified: aspartic acid, glutamic acid, asparagine, glutamine, serine, threonine, glycine, alanine, valine, proline, arginine, isoleucine, leucine, tryptophan, phenylalanine, cysteine, ornithine, lysine, histidine and tyrosine (Ribeiro et al., 2008).

To our knowledge this is the first study to identify the presence of so many amino acids for wild edible mushrooms species, confirming the importance of the mushrooms in relation to their nutritional value.

Of all the species studied, it can be concluded that *Boletos edulis* followed by *Tricholoma equestre* are those with the highest amounts of amino acids.

The mentioned study concluded that *Tricholoma equestre* is one of the most interesting mushroom species. At the *Tricholoma equestre* and in the other mentioned species, the compound that is present in larger amount is alanine. Alanine is an important nonessential amino acid, indispensable in the metabolisms of glucose and tryptophan, causing cholesterol-

lowering effects in rats. Glutamine is also one of the major compounds but not in *Tricholoma.* This amino acid is biosynthesized from glutamate and is converted to glutamic acid in the brain, being essential for the cerebral activity (Ribeiro et al., 2008).

4. Experimental Methodology

4.1. Brain dissection and Preparation of hippocampal slices

For the experiences pregnant Wistar rats, 8 to 12 weeks old, with 17 to 18 days of gestation.were used. The animals were sacrificed by cervical dislocation and then decapitation and the brain dissection were performed. The cerebral cortex was separated from the cranial and optic nerves and immediately immersed in an **ice cooled solution** (5 - 8 ºC) of artificial cerebrospinal fluid (ACSF), previously oxygenated with carbogen (95 % O_2, 5 % CO_2).

Then, the brain was placed over an icy petri dish covered with a filter paper, previously bathed with ACSF. Afterwards, with the help of a spatula and a scalpel, a cut was made between the two hemispheres and the hippocampi were separated of the remaining tissues (Figure 6). Throughout this process, the hemispheres were constantly wet with icy ACSF .

Each hippocampus was put dorsally over the same petri dish and transversal slices, with a thickness of 400 μm, obtained from the middle one third region of the hippocampus. The slices were cut with an instrument with six parallel blades and six separators. The slices were removed from the instrument with the help of a fine brush and placed in another ACSF solution, oxygenated with carbogen (95 % O2, 5 % CO2), at room temperature.

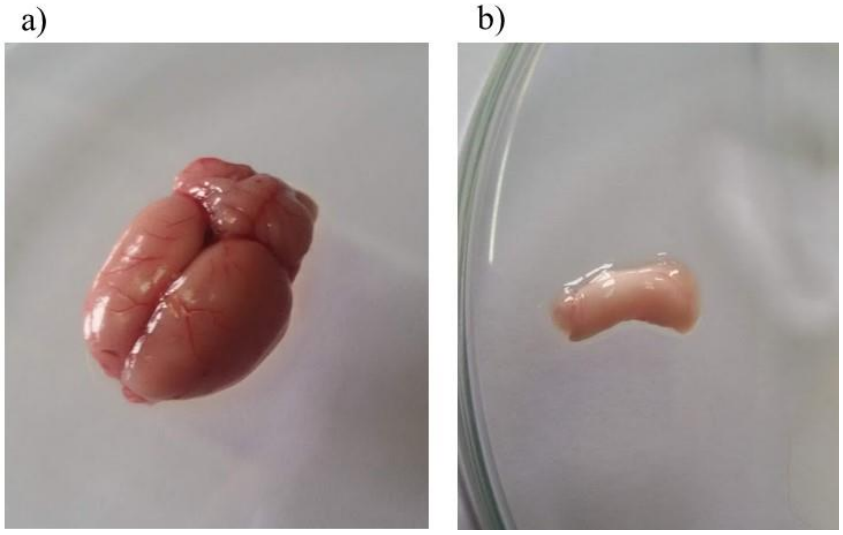


Figure 6: Wistar rat brain (a) and hippocampus (b).

4.2. Experimental arrangement and measurement of optical signals

The experimental setup used for the acquisition of the optical ROS signals, consisted of a microscope (Zeiss Axioscop) containing an halogen/tungsten light source (12 V, 100 W), as shown in Figure 7.

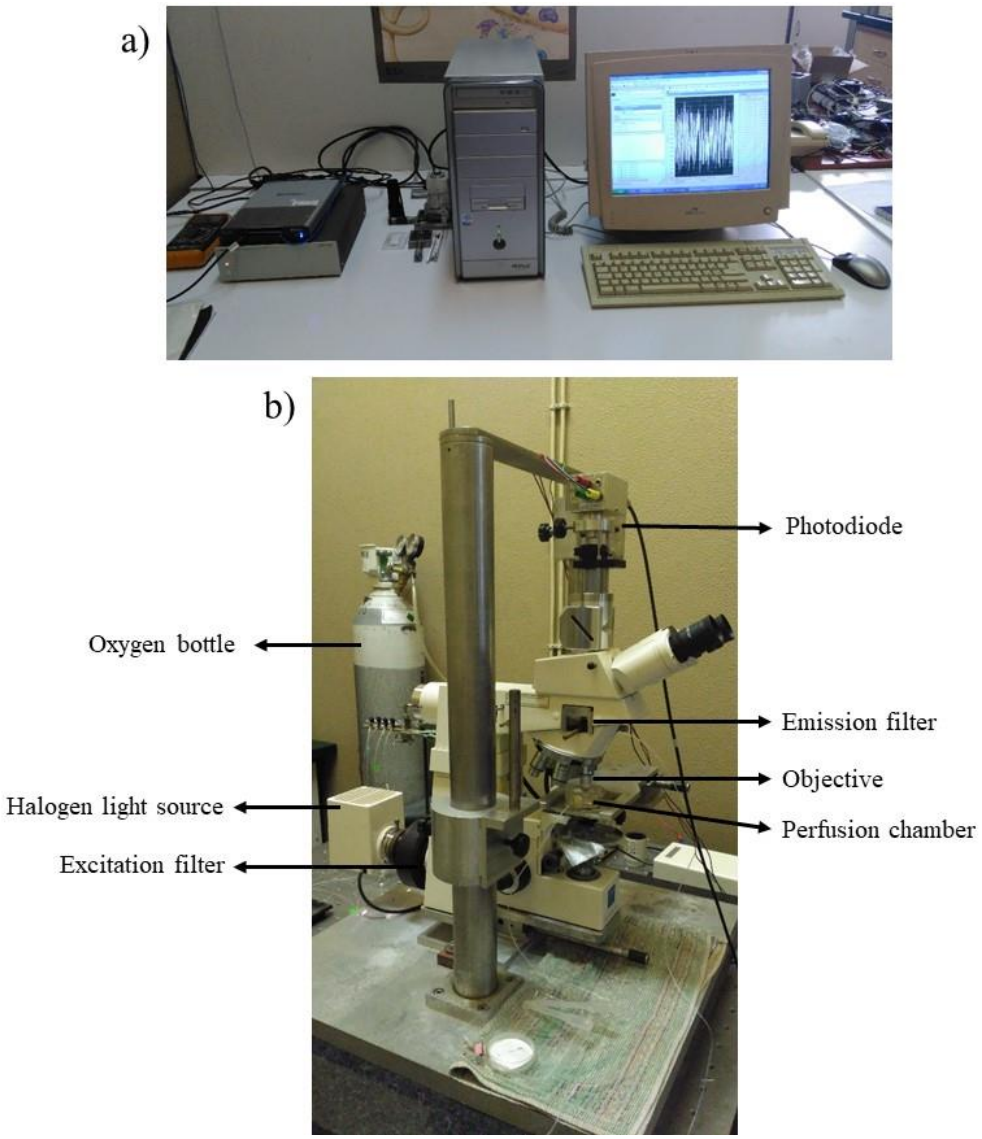


Figure 7: a) Data acquisition system; b) Experimental arrangement used in the measurement of optical signals.

The light emitted by the slice inserted in the experimental chamber located on the microscope, which had a transfluorescence arrangement, was captured by means of a water immersed lens (40x, N.A., 0,75), with a working distance of 1.6 mm. An excitation filter of 480 nm, with a 10 nm bandwidth and a high-pass emission filter of 500 nm, allowed the

selection of the light emitted in the visible region preventing contamination caused by incident light.

The fluorescence signals were detected using a **silicon** photodiode (Hammamatsu-S1226-Serie, with an area of 1 mm^2), being the signal from the photodiode converted through a current to voltage (I/V) unit with a 1GΩ feedback resistance, connected to an AC amplifier (gain 1000) and a cut-off frequency of 1 Hz. Subsequently, the signals were digitally processed by means of a 16-bit analogue/digital converter (National Instruments), at a 0,03 Hz frequency, shown in Figure .. The data wereprocessed by a digital platform through the software National Instruments Signal Express 2013®.

A peristaltic pump was used for the perfusion of liquid in the chamber (Gilson, Minipulse-3), allowing the circulation of extracellular medium, at a constant rate, between 1,5 and 2 mL/min**,** at a temperature of about 32 ºC.

4.3. Measurement of ROS optical signals

In order to analyze the effect of the polysaccharides and phenolic extracts in the formation of neuronal ROS, the hippocampal slices were incubated with the fluorescent ROS indicator H_2DCFDA. The incubation was made placing the slices in 20 mL of ACSF solution containing 2 µL of a stock solution of the H_2DCFDA indicator, giving .. M of the indicator, and these solution was continuously oxygenated with 95% O_2 and 5% CO_2. Then, the slices were transferred back to the ACSF solution.

After the incubation, the slices were perfused at a rate of 1.5 to 2 mL/min with ACSF at 36-37 ºC, in an experimental chamber coupled to a fluorescence microscope (Zeiss Axioskop).

In the chamber, the slices were placed so that the incident light was focused on CA3 hippocampal area, which contains the mossy fiber synapses. The ROS signals were collected with a sampling frequency of 1,66 Hz, every 1-minute interval for different periods of time, each of the signals being the average of each 100 consecutive data set plotted at 1 min intervals. To collect these data, during the initial 30 minutes the slices were perfused with the ACSF medium and afterwards, for 30 min, with a polysaccharides or phenolic compounds solution, in one of the used concentrations. Finally, ACSF was reintroduced for 30 minutes.

In each graph, the first 10 points, corresponding to 10 minutes of data collection, form the baseline. In this study the autofluorescence component, due to the intrinsic fluorescence of

mitochondrial proteins was considered constant. Since all signals were normalized by the average of the 10 baseline data points, the plotted data are the same with or without subtracting the autofluorescence component. This component was obtained from an equivalent region of non-incubated slices and is, on average, 0.7 of the measured (total) fluorescence of each baseline point (Neves, 2018)

4.4. Origin of the mushroom samples

The mushroom samples of *Tricholoma equestre* were collected in Winter of 2016, at a forest in the region of Cantanhede (Portugal). Posteriorly, the mushroom were cut in fragments and frozen and the remaining procedure and extraction of Polysaccharides and Phenolic compounds was performed in Department of Agronomy, UTAD, Vila Real.

4.5. Solutions

4.5.1. ACSF solution

To simulate the neuronal extracellular environment, the artificial cerebrospinal fluid (ACSF) solution was prepared at the beginning of each day with experiments (Figure 8). This solution was prepared using four liquid stock solutions (KCl, $MgCl_2$, $CaCl_2$ and $CaCl_2.2H_2O$) at 1M concentration, kept in the refrigerator, and three other solid compounds. The ACSF solution consists of the following compounds with the mentioned concentrations: 3,5 mM of KCl; 2 mM of $MgCl_2.6H_2O$; 1,25 mM of $NaH_2PO_4.2H_2O$ and 2,0 mM of $CaCl_2.2H_2O$, 124 mM of NaCl; 24 mM of $NaHCO_3$ and 10 mM of D-glucose ($C_6H_{12}O_6$). D-Glucose was the last compound to be applied, to prevent the solution from becoming cloudy.

The pH of this solution was approximately 7.4. Finally, after stirring, the solution was oxygenated for 10 min with carbogen (95 % O2 and 5 % CO_2).

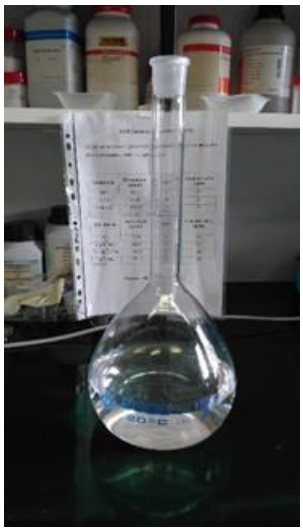


Figure 8: ACSF solution.

4.5.2. H_2DCFDA

The concentration of the stock solution of the ROS indicator H_2DCFDA was obtained by dissolving 9.74 mg of the indicator in 2 mL of DMSO. The slices were incubated in 20 mL of ACSF solution containing 2 µL of a stock solution (20 mM) of the H_2DCFDA indicator.

4.5.3. Polysaccharides solution

For the preparation of the polysaccharides solutions, the solid fraction of the polysaccharide extract from the *Tricholoma equestre* mushroom species was added to ACSF (Figure 9). The solid extract was previously ground with the aid of a mortar, to reduce the particle size. Afterwards the mixture was homogenized and was used to prepare three different concentrations (1 $g.L^{-1}$, 0.5 $g.L^{-1}$ and 0.1 $g.L^{-1}$).

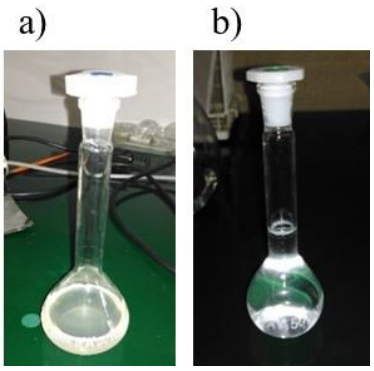


Figure 9: Polysaccharides solution: a) 1.0 g.L-1 and b) 0.1 g.L-1.

4.5.4. Phenolics solution

The preparation of the phenolic compounds solutions, was similar to that of the polysaccharides. Briefly, the solid extract from the *Tricholoma equestre* mushroom was, after being ground, added to ACSF. The mixture was then homogenized and used in the preparation of the same three concentrations (1 $g.L^{-1}$, 0.5 $g.L^{-1}$ and 0.1 $g.L^{-1}$).

Since, after homogenization the mixture was still not completely dissolved, the solutions were filtered to leave a more homogenous medium. It can be concluded that these solutions did not have exactly the desired concentration, but a slightly lower one, since the amount of lost solid residues was quite small (Figure 10).

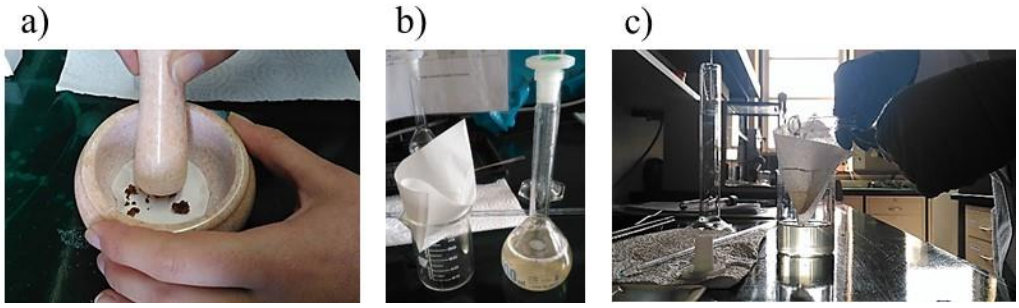


Figure 10: Preparation of the phenolics solution. a) Grinding of mushrooms extract; b) Phenolics solution before filtration; c) Filtration of the phenolics solution.

4.5.5. 4-Hydroxibenzoic acid solution

The 4-hydroxibenzoic acid is a phenolic compound existing in the Tricholoma equestre species. The solution (1 g.L-1) was prepared adding powdered 4-hydroxibenzoic acid to ACSF.

The polysaccharide and acid solutions were sufficient to perform the experiments in triplicate (n = 3). However due to the low amount of phenolic compounds, the experiments for this compound were made in duplicate.

4.6. Chemicals products used

The products used to carry out the experiments were obtained from:

Life Tecnologies, Canada

- H_2DCFDA

Sigma-Aldrich Química, S.L., Sintra:

- D-glucose;
- KCl;
- NaCl;
- $NaH_2PO_4.2H_2O$;
- $NaHCO_3$;
- $CaCl_2.2H_2O$;
- 4-Hydroxibenzoic acid

Merck Chemicals, Germany:

- $MgCl_2.6H_2O$

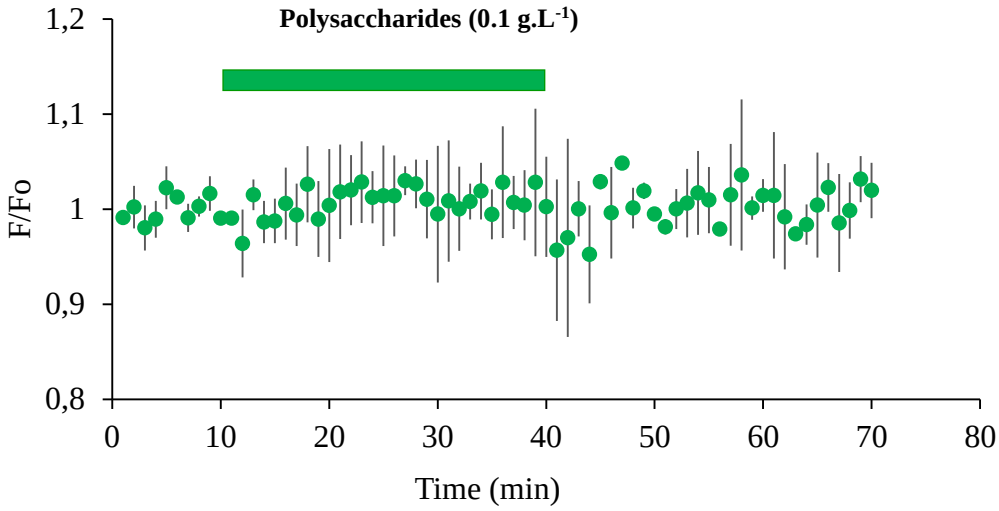
5. Results and Discussion

5.1. Effect of polysaccharides in neuronal ROS studies

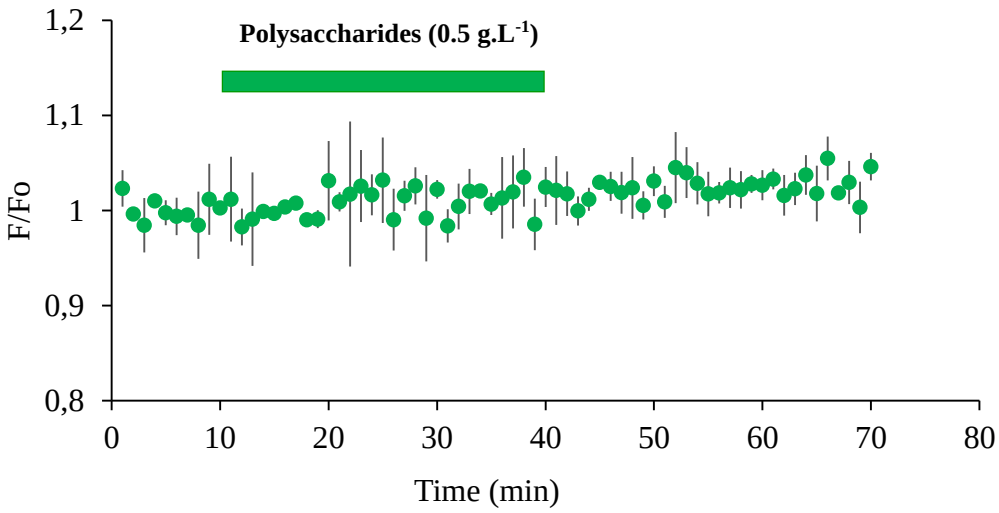
This work was performed using polysaccharides and phenolic compounds that were extracted from the *Tricholoma equestre* mushroom species. The studies were designed to evaluate the effect of different concentrations of those compounds on neuronal ROS signals. These were detected at the mossy fiber synapses from hippocampal CA3 area, using the fluorescent ROS indicator H_2DCFDA.

Figure 11 shows the effect of three different concentrations (0.1, 0.5 and 1 $g.L^{-1}$) of the polysaccharides extract on the neuronal ROS signals. The graphs show normalized fluorescence values plotted as a function of time, at 1 min intervals, having the compounds been perfused during 30 min. The rest of the time, both before and after their application, the slices were exposed for the same period, 30 min, to the artificial cerebrospinal medium (ACSF). In all cases the baseline was obtaind from the last 10 data points of the initial ACSF perfusion.

a)


Polysaccharides (0.1 g.L-1)
1,2
1,1
1
0,9
0,8
F/Fo
0
10
20
30
40
50
60
70
80
Time (min)

b)


Polysaccharides (0.5 g.L-1)
1,2
1,1
1
0,9
0,8
F/Fo
0
10
20
30
40
50
60
70
80
Time (min)

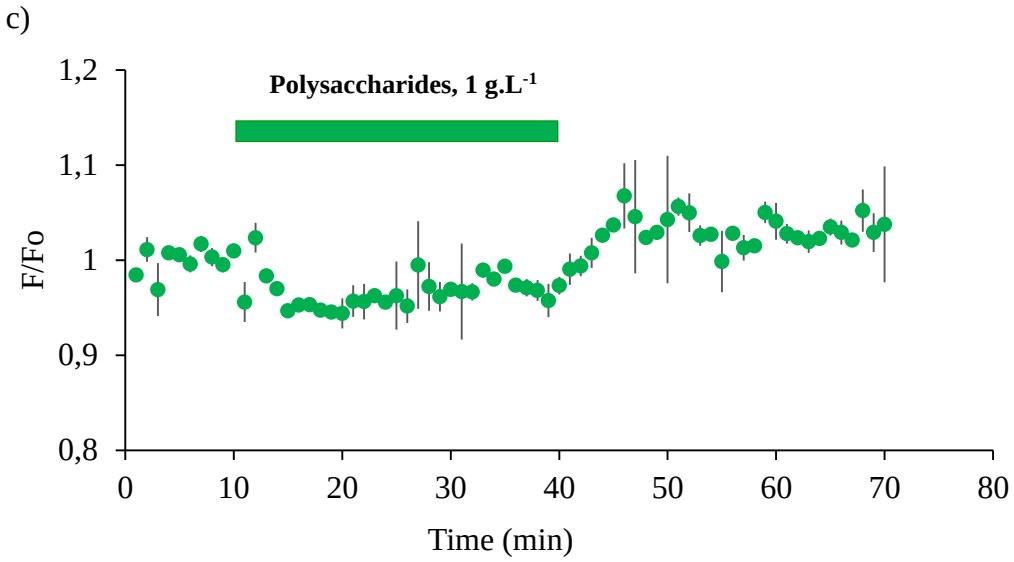


Figure 11: Neuronal ROS changes induced by the polysaccharides mixture extracted from the *Tricholoma equestre* mushroom. The signals were obtained at the concentrations of a) 0.1 g. L^{-1} (n = 3), b) 0.5 g. L-1 (n = 3), c) 1 g.L-1 (n = 3). The bars represent the period of application of the solutions. All values were normalized by the average of the first 10 responses and represent by the mean ± SEM. F, fluorescence; F0, basal fluorescence.

It can be observed, in Figure 11, that the application of the lower concentration, 0.1 $g.L^{-1}$, did not cause significant changes with respect to the baseline, represented by the first 10 points of the graph, or following the compounds removal, during the last 30 min (n = 3). Similar results, i.e. no clear changes, were obtained with the intermediate concentration of 0.5 $g.L^{-1}$ (n = 3), although it is five times higher than the first one.

Applying an even higher concentration, 1 $g.L^{-1}$, led then to the observation of ROS changes that were reduced with respect to baseline, by 3 ± 1% (n =3), in the period 35-40 min. This result indicates that the polysaccharides solution had an impact in the amount of ROS formed, leading to its reduction, thus attenuating their possible negative effects. In the final 30 min, during the ACSF reperfusion, the signals returned to the baseline level indicating that the polysaccharides effect was reversible.

To compare the amplitudes of the variations induced by the different concentrations of the polysaccharides, a bar chart was represented by figure 12, in the final 5 min of the perfusion.

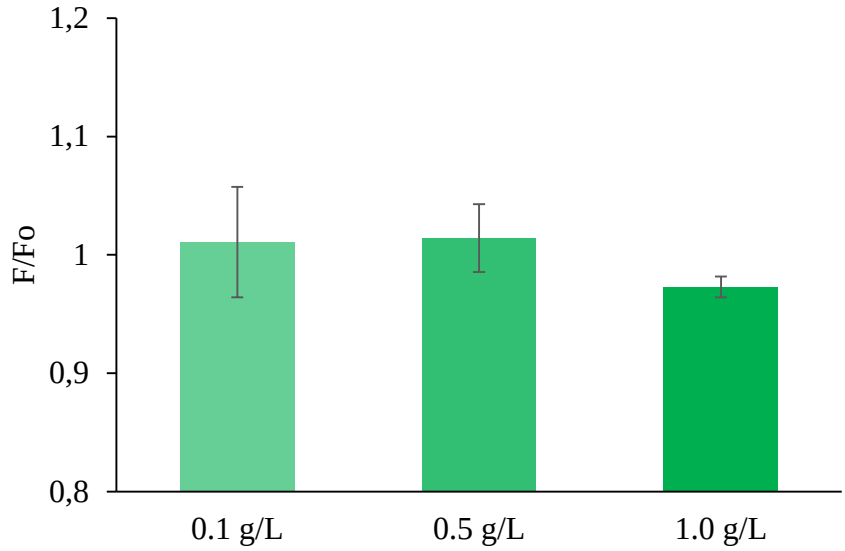


Figure 12 - Normalized amplitude of ROS signals evoked by different concentrations of the polysaccharides extract. The amplitude of the bars represents the average ± SEM of the last five data points obtained in each medium. F, fluorescence; F0, basal fluorescence.

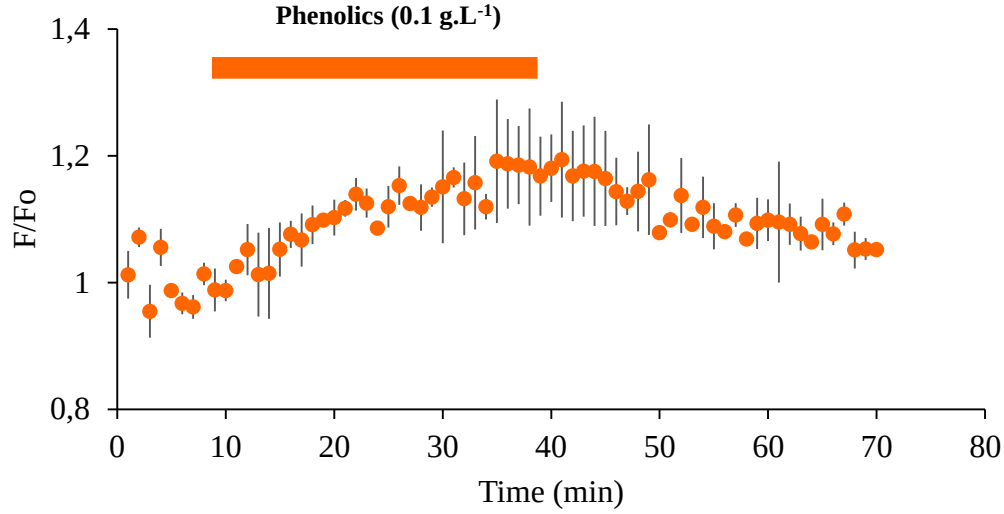
The polysaccharides for their antioxidant properties have the function of scavenging ROS signals, increasing their performance in antioxidant processes such as SOD, CAT and GPx (Kozarski et al., 2015)(Korkmaz, 2018).

These results are in agreement with the conclusions of this study, with an increase in the enzymatic antioxidant activity leading to a decrease in the ROS signals and the attenuation of its negative actions.(Kozarski et al., 2015)(Korkmaz, 2018).

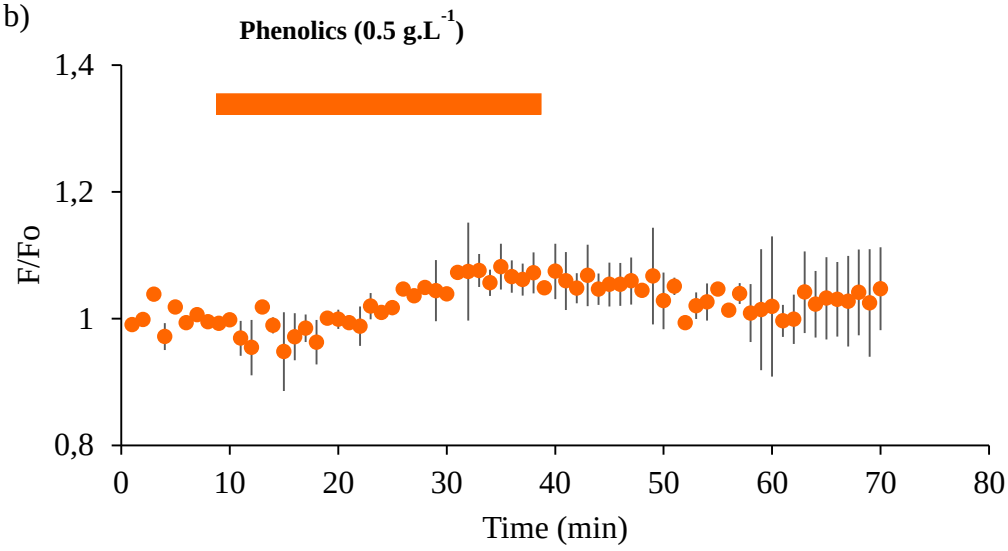
5.2. Effect of phenolic in neuronal ROS studies

The next set of experiments evaluated the effect of the phenolic compounds extract in the fluorescence ROS signals. The results can be seen in Figure 13 for the same concentrations (0.1, 0.5 e 1 $g.L^{-1}$) that were used before. Again, in all experiments, the slices were perfused for equal periods of time (30 min) and sequentially with ACSF, the media of interest and ACSF. As in the previous figure, the baseline corresponds to the last 10 min of the first ACSF application.

a)


Phenolics (0.1 g.L-1)
F/Fo
1,4
1,2
1
0,8
0
10
20
30
40
50
60
70
80
Time (min)

b)


Phenolics (0.5 g.L-1)
F/Fo
1,4
1,2
1
0,8
0
10
20
30
40
50
60
70
80
Time (min)

c)

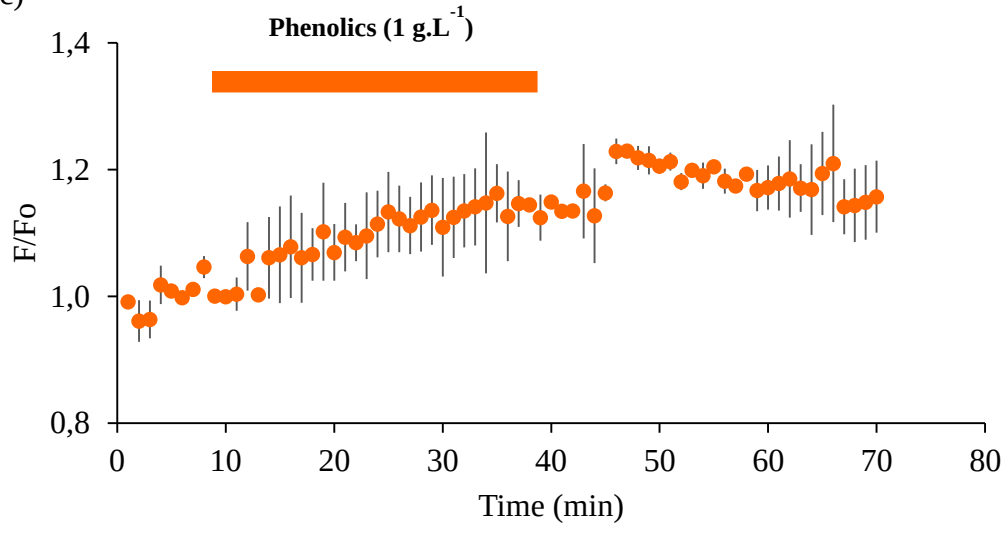

Phenolics (1 g.L-1)
F/Fo
1,4
1,2
1,0
0,8
0
10
20
30
40
50
60
70
80
Time (min)

Figure 13: Neuronal ROS signals evoked by the solutions containing the polyphenols mixture extracted from the *Tricholoma equestre* mushroom. The signals were obtained at the concentrations a) of 0.1 g.L-1 (n = 2), b) of 0.5 g. L-1 (n = 2) and c) of 1 g.L^{-1} (n = 2). The bars represent the period of time during which the solutions were perfused. The average of the first 10 responses was used to normalize all data points that are represented as the mean ± SEM. F, fluorescence; F0, basal fluorescence.

The upper part of the figure (Fig 13a) reveals that circulating the 0.1 g.L^{-1} solution induced an accentuated increase in the ROS signals, with an amplitude of 18 ± 7 % (n = 2) above the baseline, in the same time interval (35-40 min). Upon ACSF reperfusion the signals decreased reaching almost the baseline level, i.e.7 ± 2%, in the last 5 minutes.

The panel relating to Fig 13b) represents the data obtained with the 0.5 g.L^{-1} medium that led to a small increase in the ROS signals, with an amplitude of 6 ± 3 % (n =2, 35-40 min). Following removal of the phenolic solution the signal started to recover but remained at a level higher than that of the baseline, measuring 3 ± 7 % in the period 65-70 min.

The application of the higher concentration, 1 g.L^{-1} (Fig. 13c), caused also an enhancement of the ROS signals, of 14 ± 3 % (n = 2) at 35-40 min, but instead of initiating the recovery immediately upon washout the signals increased further decaying afterwards to a high level. Thus, in this case, at the end of the ACSF reperfusion, in the time interval 65-70 min, the signals maintained a large amplitude, of 17 ± 6 %, and were not reversible.

In Fig 13c, during the exposition of the slices to the 1 g.L^{-1} medium, the signals clearly increased reaching a maximum of 21 ± 2 % in the presence of the phenolics.

5.3. Effect of phenolic compound 4-Hydroxybenzoic acid in neuronal ROS studies

One study indicates that the 4-hydroxybenzoic acid is one of the phenolic compounds that exist in the *Tricholoma equestre* mushrooms and suggests that it is the most abundant (Ribeiro et al., 2006). For these reasons, a synthetic form of that compound was also tested with respect to the effect on ROS signals In Figure 14 the profile of 4-Hydroxybenzoic acid was represented.

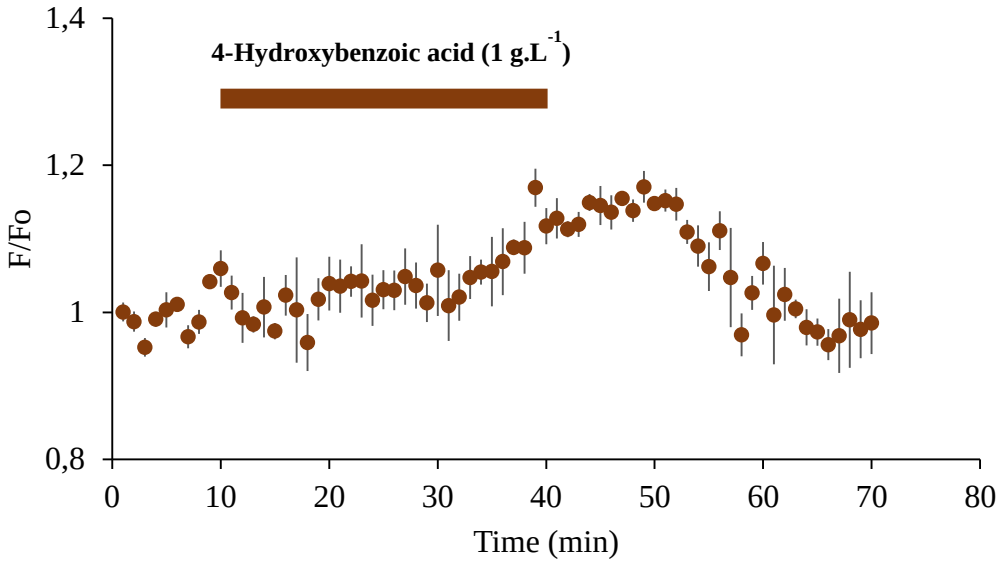


Figure 14: Time course of the ROS signals induced by the medium prepared with the synthetic phenolic 4-hydroxybenzoic acid. The data were obtained for a concentration of 1 g.L^{-1} (n = 3). The bar represents the time during which the solution was perfused. The average of the first 10 responses was used to normalize all data points which are represented as the mean ± SEM. F, fluorescence; F0, basal fluorescence.

The experimental protocol followed was similar to that previously applied. In the presence of the 1 g.L^{-1} of acid solution, the ROS data behaves as for the 1 g.L^{-1} extract, increasing by 9 ± 3 % (n = 3, 35-40 min) and even more, by 14 ± 2 % (45-50 min), following washout. However, in this case, the signal recovered fully.

To compare the amplitudes of the variations induced by the different concentrations of the phenolic compounds solution and the acid 4-Hydroxybenzoic acid, a bar chart was represented by figure 15, in the final 5 min of the perfusion.

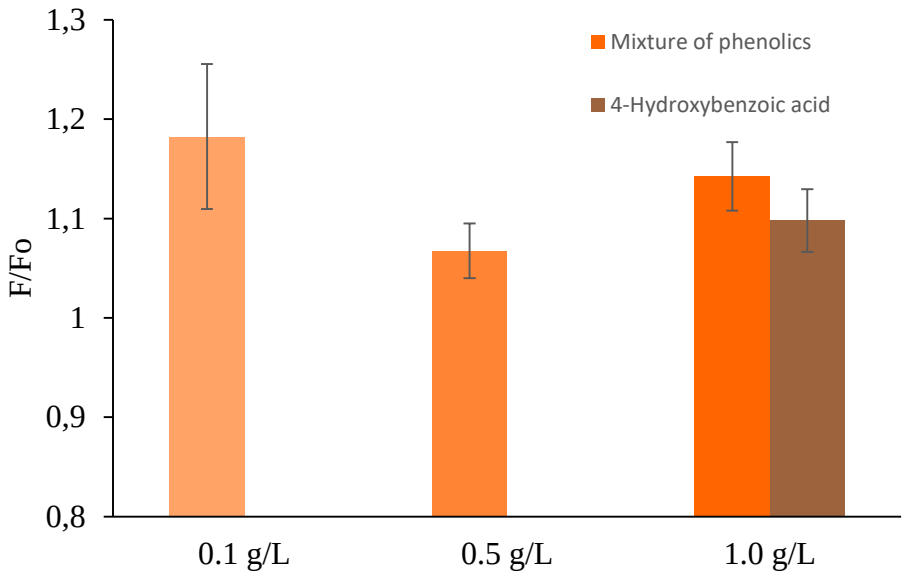


Figure 15 - Amplitude of ROS signals induced by the three concentrations of the phenolic compounds mushroom extract and by the 4-hydroxybenzoic acid. The amplitude of the bars represents the average ± SEM of the last five data points recorded in each solution. F, fluorescence; F_0, basal fluorescence.

Taking into account the study of resveratrol and curcumin performed by Kosarsky, it is also likely that similar processes occur with mushroom polyphenols, requiring special attention to possible harmful redox effects of mushrooms. The pro-oxidant properties of polyphenols may explain the results obtained in this work, since, instead of an anti-oxidant behavior they lead to an increase in ROS production in the presence of the mushroom polyphenols. Thus, it would rather interesting to perform more studies on this issue using polyphenols from the *Tricholoma equestre* species(Kozarski et al., 2015) (Bilski et al., 2000).

6. Conclusions and Future Work Perspectives

This master`s thesis project focused in on the study of the *Tricholoma equestre* mushroom species based on the investigation of the effect of polysaccharides and phenolic compounds on the production of ROS, in neuronal cells.

For the studies carried out with extracts of polysaccharides, the concentration of 1 g. L^{-1} showed the greatest variation in ROS signals, compared to concentrations of 0.1, 0.5 and 1 g. L^{-1}, which did not present significant variations. Thus, the higher concentration induces a decrease in the ROS signals, with an amplitude in the order of 14%, at the time interval of 35-40 min, thus evidencing the antioxidant properties of the polysaccharides.

The results obtained with the phenolics compounds indicated an increase in ROS signals being that for the concentration of 0.5 g. L^{-1} the response variation wasn`t as evident (6 %) as the one observed for the concentrations of 0.1 and 1 g. L^{-1} (18 and 14 %, respectively).

The last group of experiments was made with the synthetic compound, 4-hydroxybenzoic acid (1 g.L^{-1}). The results for this compound indicated similarities to the ones made to the other phenolic extracts, with a maximum value of about 14% in amplitude, upon the acid removal.

In the future, it would be interesting to extract more polysaccharides and phenolic compounds from the *Tricholoma equestre* mushroom species, in order to carry out new studies of the ROS signals, applying different concentrations of solution, in order to increase the number of experiments, to complement those made in this study.

It is also important to make experiments for longer time intervals, in order to more accurately perceive the behavior of the upon washout profile. In addition, studying the behavior of phenolic compounds in the performance of ROS signals for other species of mushrooms, will be an added value to evaluate the similarity in relation to the species *Tricholoma equestre.*

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Attachments

APPENDIX I

Table I.1: Safety and risks associated to the reagents used to prepare all the solutions

	Suplier	Product number	CAS number	H-Phrases	P-Phrases	Hazard pictograms	Signal word
4-Hydroxybenzoic acid	Sigma-Aldrich Química, S.L.	H20059	99-96-7	H318 H335	P280 P305 + P351 + P338 + P310		Danger
2`,7`- dichlorodihydrofluorescein diacetate (H$_2$DCFDA)	LIFE TECHNOLOGIES EUROPE BV	D399	N/A	N/A	N/A	N/A	N/A
$CaCl_2.2H_2O$	Sigma-Aldrich Química, S.L.	223506	10035-04-8	H319	P305 + P351 + P338		Warning
D-Glucose	Sigma-Aldrich	G8270	50-99-7	N/A	N/A	N/A	N/A

	Química, S.L.						
KCl	Sigma-Aldrich Química, S.L.	P9541	7447-40-7	N/A	N/A	N/A	N/A
$MgCl_2.6H_2O$	Merck KGaA	105833	7791-18-6	N/A	N/A	N/A	N/A
NaCl	Sigma-Aldrich Química, S.L.	746398	7647-14-5	N/A	N/A	N/A	N/A
$NaH_2PO_4.2H_2O$	Sigma-Aldrich Química, S.L.	71505	13472-35-0	N/A	N/A	N/A	N/A
$NaHCO_3$	Sigma-Aldrich Química, S.L.	792519	144-55-8	N/A	N/A	N/A	N/A
Distilled water	RephiLe Bioscience, Ltd.	N/A	N/A	N/A	N/A	N/A	N/A
Ultrapure water	RephiLe Bioscience, Ltd.	N/A	N/A	N/A	N/A	N/A	N/A

APPENDIX II

Figure II.1: Procedure for obtaining the hippocampus slices.