Università degli studi di Genova

Department of Experimental Medicine DIMES

PhD course in Experimental Medicine

Cycle XXXV

Curriculum: biochemistry

EXTRACTION, ANALYSIS AND CHARACTERIZATION OF PRIMARY AND SECONDARY METABOLITES OF THE BASIDIOMYCETE *PLEUROTUS OSTREATUS* (Jacq.) P. KUMM., 1871, USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TECHNIQUES COUPLED WITH MASS SPECTROMETRY AND THEIR POSSIBLE USE FOR HUMAN HEALTH

Tutor Professor

Gianluca Damonte

PhD candidate

Mirko Benvenuti

Academic Year 2019/2020

to research, curiosity, knowledge and science

1. INTRODUCTION

1.1 Why fungi?

Fungi are complex organisms and still, for many aspects, little known and studied. Biochemically, fungi are much more similar to animals than to plants and indeed, as sessile organisms, they are an interesting model on which to work. In recent decades, the increasing problems related to food production and waste management, are driving the agricultural activities to optimize and exploit resources in circular economy perspective focused at reducing and optimizing the production of waste. Several companies and researchers have carried out studies aimed at achieving these goals. For example, it has been demonstrated that the lavender and lemon balm waste contain exploitable natural biologically active compounds [1] and can be recovered and utilized for the production of a variety of foodstuffs. Chemically, these compounds are mainly polyphenols and aroma substances, remained in the waste. Phenolic compounds have been proven to exhibit numerous beneficial biological activities including antioxidant, antimicrobial, insecticidal, anti-inflammatory, anti-carcinogenic, etc. [2; 3]. The ecological role of Fungi in natural trophic chains suggests that these organisms can be exploited fruitfully in circular economy perspective to recycle and valorise vegetal waste derived from agriculture. In last years the number of fungal species exploited for food, nutraceutical products and cosmetics has increased. However, *P. ostreatus* today is still one of the most cultivated edible mushrooms worldwide [4]. Despite the extensive literature on *P. ostreatus*, many aspects regarding its cultivation, such as the cultivation techniques or the influence of different substrates on the biochemical profile of sporomata, still need to be investigated. The lavender (*Lavandula angustifolia*) is a widely utilized crop in the

essential oil industry. Bulgaria, France, UK, China, Ukraine, Spain, and Morocco are the biggest worldwide producers. Bulgaria, in particular, has recently become the world's major producer with 100 tons lavender oil per year. During the extraction process the lower quantity of essential oil in the lavender (0.8–1.3% / fresh plant) results in enormous quantities of solid residues which still has a high content of useful substances (dozen of thousands of tons worldwide). These wastes are usually discarded directly in the nearby locations or disposed as special waste leading an environmental issue. The aim of our project focuses on the identification and qualitative-quantitative characterization of fungal primary and secondary metabolites of interest for human health, extracted from the species *Pleurotus ostreatus*, and analyzed by high performance liquid chromatography (HPLC) coupled with mass spectrometry at medium and high resolution to try to identify molecules with potential pharmacological interest. The research has been focused on the analysis of molecules extracted from two different strains of the fungus *Pleurotus ostreatus* grown on enriched or not enriched substrates; for the non-standard substrates, the enrichment consists of exhausted waste produced from the extraction of essentials oils from lavender. The extraction of the metabolites from fungi, for a subsequent analysis, has been carried out with several modalities, initially taken from the bibliography available on the topic, and various solvents or solvent mixtures, under different conditions of sample preparation (temperature, pH, degree of polarity of solvents, etc.). This screening operation allowed to identify the most suitable procedure to extract as many molecules as possible produced by the mushroom itself, even starting from different parts of the mushroom. In this context, the sample preparation was the basis on which to build the reference standard for any future research. The setup of an appropriate

high performance liquid chromatographic separation allowed the separation of each single component for its subsequent specific structural characterization. This last part was also initially conducted through the aid of the scientific literature available to date. So, the mass spectrometer represents one necessary tool both for the possible resolution of partially fused chromatographic peaks and for the identification of the molecular mass of the compounds of interest and for their characterization. In this context, obtaining chromatographic and spectral images allowed to get the fingerprint of the metabolites that represented an extremely useful starting point to evaluate the direct effect of the different culture media on the metabolomic pattern expressed by the examined mycetes. Indeed, HPLC coupled with mass spectrometry is a powerful analytical technique that can give numerous information regarding molecules' structure from samples present in extremely small amounts. Therefore, the project sought to identify the molecules extracted from the fungal species and to identify the differences between the production of primary and secondary metabolites in different strains of *Pleurotus ostreatus* in relation to growth conditions. Through the analysis of samples collected on different substrates and the identification of the molecules produced by the fungus, this approach allowed the identification and characterization of the metabolites and their qualitative and quantitative differences, where present. The cultivation of previously selected macrofungus species may lead to the eco-recycling of "difficult to dispose" substrates, produced by other entities involved in the project. To summarize, the research project has been focused on the following main points: i) to improve the sensitivity of the HPLC coupled with mass spectrometry analysis; ii) to identify molecules of interest in relation to their biological activity and potential benefit for cellular health extracted from *Pleurotus ostreatus* and

characterized using HPLC coupled with mass spectrometry; iii) to identify differences between the production of secondary metabolites in different strains of *Pleurotus ostreatus* in relation to the conditions of growth; iv) to evaluate the potential beneficail biological activity of *Pleurotus* extracts regarding protection against external agents and oxidative stress extracts on different cell models. The analyses in HPLC coupled with mass spectrometry carried out on our samples, harvested on different substrates, with increased concentration of exhausted waste of lavender, have resulted in the identification of many different molecules, in both domesticated and wild strain, belonging to different families of molecules considered as secondary metabolites. The potential use of natural secondary metabolites produced by fungi should be considered the new frontier to find molecules with powerful beneficial effects and should be studied more and more thoroughly to meet the demand for drugs and supplements of natural origin.

1.2 Aim of the work

Oxidative stress is one of the main causes of premature cellular aging which can be at the origin of numerous acute or chronic diseases that arise with age. New natural sources of molecules with powerful antioxidant properties are one of the possible solutions in the formulation of antioxidant preparations for protective and anti-aging purposes. As harvested fungi are an evergrowing industry the characterization of their primary and secondary metabolites and their experimentation on cell cultures are fundamental prerequisites for the identification and subsequent experimentation of molecules of cosmetological, pharmaceutical and / or nutraceurtic interest. The aim of this work was the development of a liquid chromatography method at high pressure /

performance coupled with mass spectrometry that allowed the simultaneous analysis of as many primary and secondary metabolites extracted from Pleurotus ostreatus as possible. The metabolites identified were tested on cell cultures to highlight their possible cytotoxicity and their possible applications for human health.

2. FUNGI

2.1 What are fungi?

The kingdom of fungi, also called mycetes, includes numerous unicellular and multi-cellular eukaryotic organisms, characterized by a unique type of trophic fungi. Mushrooms are, in fact, heterotrophic organisms by absorption: they release, at the hyphal level, digestive enzymes into the growth environment to predigest complex biological molecules such as cellulose to simpler, smaller and more easily absorbed molecules. The main enzymes that are released are endocellulases, exocellulases and laccases. Mushrooms have been classified for a long time as higher plants, but currently it is believed that they derive from an ancestor common to the metazoans, from which they were then separated; for this reason, they represent a kingdom of their own. Mushrooms are distinguished from the kingdom of plants and animals for peculiar characteristics; more precisely, there are three elements characterizing the mycetes: heterotrophy; lack of differentiated tissues and of conductive elements such as sap; spore reproduction. The kingdom includes more than 120,000 known species, although the diversity has been estimated between 2.2 and 3.8 million species [5] and probably much more. Among these we find both very simple unicellular organisms, such as yeasts, and multi-cellular organisms, such as mushrooms that are commonly consumed as food. The vegetative body of the fungi is called thallus, which, in the multi-cellular species, is generally filamentous and composed of filaments called hyphae. Mushrooms do not have differentiated cells in tissues, hyphae organize themselves to form an intertwined lattice work called mycelium and this organisation in pseudo tissues is one of the main reasons as they should be considered more as model organisms. The walls that delimit the thallus of a mushroom are very thin and

have a complex layered structure; these are equipped with fibrillar polymers inside and amorphous polymers to form the matrix. The main component is chitin, a polymer of N-acetylglucosamine, organized in microfibers. Other components are the beta-glucans and chitosan. In yeasts, chitin is scarcely present, therefore the mannans prevail. It is also absent in oomycetes, where a cellulose-like polymer prevails, organized not in fibers but in an amorphous matrix, as polysaccharide in algae. As the animals, mushrooms produce glycogen. As eukaryotes, the DNA of the fungi is enclosed in a membrane bounded nucleus. Mushrooms also have cytoplasmic organelles bordered by membranes, such as mitochondria and vacuoles, and 80S ribosomes. Differences from other eukaryotes were found, for example, in the spatial organisation of tubulins (a condition unique among other eukaryotes) [6]. Unlike organisms belonging to the plant kingdom, fungi do not possess chloroplasts and, for this reason, do not photosynthesize. All mushrooms are heterotrophic, can take small molecules in solution by absorbing them from the wall, but can also externally degrade more complex molecules through the secretion of extracellular digestives enzymes, with a particular production of enzymes with depolymerase action such as endo- and eso-cellulases and laccases, so as to favour the subsequent absorption. This ability makes mushrooms the most important decomposers in many environments, fundamental in ecological systems. Depending on the carbon source from which they feed, the fungi can be saprophytes, symbiont or parasites. In addition to carbon, the other three main elements they need to grow are: nitrogen, which can be assimilated by nitrates, nitrites or ammonium; phosphorus, which can be obtained either by solubilization of inorganic phosphates and by liberation through digestive enzymes that act on organic material; iron, captured from the external environment thanks to

chelating compounds called siderophores. Almost all fungi obligate aerobe: in the presence of atmospheric oxygen, they produce respiratory energy. Some facultative anaerobic species, under conditions where oxygen is scarce, can divert their metabolism towards fermentation processes, which are widely exploited by man in the food industry. The primary metabolism of the fungi is associated with a secondary metabolism, which leads to the production of molecules not essential for growth, but which can serve as protection systems against external agents, such as antibiotics, toxic substances or mycotoxins, sexual hormones. Thousands of secondary metabolites have been described in mushrooms, whose functions are still being studied and whose production tends to be specific at the level of genus, species or even strain. Fungi are an interesting model for biochemistry and metabolomics studies; in particular, as sessile organisms lacking for obvious reasons avoidance behaviour, they are in direct and constant interface with the substrate, as a source of nutrients and environment and minimal variations in the composition of it could make significant changes in their metabolomic profile.

2.2 The role of fungi in human history and health

Mushrooms, since the most ancient history, have always aroused great interest in mankind, partly because of the peculiarity of their living, partly because they have been used for various purposes, food in the foreground, but also to carry out ceremonies in which witchcraft had a preponderant role. Mushrooms are linked to man, to his diet and to the medicine since ancient times. Our ancestors used mushrooms as medicine for thousands of years. The Greek doctor Hippocrates, around 450 B.C., classified the Mushroom of the Bait (*Fomes fomentarius)* as a powerful anti-

inflammatory and cauterizing agent for wounds. The Alchemist Tao Hongjing, in the fifth century, described several medicinal mushrooms, including Ling zhi (*Ganoderma lucidum*) and Zhu ling (*Dendropolyporus umbellatus*). Ötzi, the Man of Ice, which lived almost 5300 years ago, was found with mushrooms (*Piptoporus betulinus*) inside his leather belts making a pouch, probably useful for the survival in the Alps of northern Italy. The first people of North America used the Ball mushrooms (genus *Calvatia*) to treat wounds [7]. Mushrooms are an important part of our diet and have been consumed for years for their taste and flavour. According to current estimates, higher fungi constitute at least 12,000 species known all over the world; of these, only 2000 are reported as edible; even more reduced, about 35, is the number of species of mushrooms that are cultivated for commercial purposes today [8]. It should be noted that cultivated fungi are mostly exclusive saprotrophic species: at the current state of the art it is no possible to produce reliable quantities of important and favoured symbiotic species such as *Boletus* spp. It is not fully known and understood the different type of symbiosis between fungi and plants and therefore it is nearly impossible to obtain cultivations of mycorrhizal plants. From a nutritional point of view, mushrooms are mainly composed of carbohydrates, with percentages varying from 20% (*Lepista nuda*) to 75% (*Boletus* spp.); glucose, mannitol and α, α-threalose are the most represented monosaccharide, and oligosaccharide, respectively; the reserve polysaccharide is glycogen, whose content in average is 5-10%; chitin is a structural polysaccharide, insoluble in water, which represents up to 80-90% in dry weight of the cell walls of fungi. The average protein content is 32.8% and the composition of the mushroom proteins seems to have a nutritional value higher than that of most plant proteins. The content of total lipids varies from 2% to 6%, with a

prevalence of polyunsaturated linoleic acid, acid monounsaturated oleic and saturated palmitic acid [9]. It is certainly interesting that the lipid fraction of fungi is a reservoir of vitamins, including vitamin D; mushrooms represent the only food of non-animal origin that contains it [10]. Mushrooms also contain high levels of phosphorus, calcium and potassium and relatively high magnesium content. Sodium levels are instead reduced, for this reason they could represent a valid alternative to vegetables for hypertensive subjects [11]. In addition to macronutrients, however, the fungi are also producers of a variety of molecules that show interesting biological functions. These metabolites have allowed to re-evaluate the role of mushrooms from a cosmetological and nutraceutical point of view. Thanks to these substances, the literature attributes to fungi immunomodulatory, anti-tumour, anti-bacterial, anti-inflammatory and antioxidant activities [12; 13; 14; 15]. The characteristics of mushrooms, associated with a growing awareness of consumers typical of recent years, with a greater care towards one's own physical, inner and outer well-being, also through the research of products that are "functional", makes them possible protagonists in the development of dietary supplements or in the production of skincare and make-up products. The cosmetics industry is constantly searching for ingredients of natural origin for their effectiveness related to lower toxicity effects. The aging of the skin, or skin aging, is a process related to the advance of time and to the general aging of the whole body, characterized by a progressive reduction function of the cells, which manifests itself with the loss of elasticity and the appearance of the wrinkles. To prevent, contrast or slow down this phenomenon, natural compounds that have antioxidant and anti-inflammatory properties are essential, being the inflammatory process at the base of skin aging. Thanks to their phenolic and polyphenolic content, different species of

mushrooms, such as *Lentinula edodes, Volvariella volvacea* and *Pleurotus ostreatus* have demonstrated important antioxidant activity [16; 17]. Polysaccharides, terpenes, phenolic compounds, sterols, fatty acids, and other bioactive metabolites isolated from numerous species were, instead, identified as potential anti-inflammatories, capable of reducing the production of phlogistic mediators [18]. In addition, to prevent skin aging and the appearance of wrinkles, there are fundamental molecules that reduce the synthesis of melanin and inhibit all activity enzymes that damage the matrix, i.e., metalloproteases, collagenases, hyaluronidases and tyrosinases [19]. Again of interest in the cosmetic and cosmeceutical field, the mushrooms possess antibacterial activities. To be able to survive in nature, like all living organisms, fungi have to fight against various stressors, including infectious processes; it is not surprising, therefore, that they have systems to combat microbial growth. The skin is constantly colonized by a large number of microorganisms, among which are mainly the recall *Staphilococcus aureus* and *Streptococcus* spp. Changes in quali-quantitative composition of the skin microbiota are at the basis of some diseases, such as atopic and seborrheic dermatitis, folliculitis, appearance of furuncles, psoriasis and others. Numerous studies have highlighted the antimicrobial potential of fungi and their bioactive compounds. Lentinula edodes has been indicated as the most interesting species against both gram-positive and gram-negative. These antimicrobial activities seem to be attributable to different molecules isolable from fungi, such as terpenes, steroids, peptides and proteins, carboxylic acids and, in some cases, ribonucleases [20; 21].

2.3 *Pleurotus ostreatus*

Fig. 1 Typical aspect of *Pleurotus ostreatus* carpophores cultivated in a controlled environment.

Pleurotus ostreatus is one of the most widely cultivated fungal species in the world. It is a species belonging to the *Pleurotaceae* family and was first cultivated in Germany as a support food during the First World War. It is a saprotrophic species that typically grows on various types of trees. It is a species with a large cap that can reach 30 cm in diameter, white to greyish in colour, a strongly off-centre and curved stem, decurrent gills and a typically white spore print. A typically saprotrophic species, it is known to produce particularly active endo- and exo-cellulases which it uses to degrade rotting wood. Often as a supplement to stock, this fungus is able to produce anthelminthic substances that kill the nematodes present in its growth environment. As one of the most cultivated mushrooms in the world and widely consumed in all countries, the

characterisation of its metabolites for the purposes of human health is certainly an interesting field of research that should be explored further.

3 MASS SPECTROMETRY

Mass spectrometry is powerful analytical technique developed at the beginning of 1900 by the experiences of J.J.Thompson, who highlighted the formation of electrons inside a vacuum tube to which a difference in electrical potential was applied. He observed that this technique could be used to analyze substances, but initially it was applied only in the field of physics, where, in particular, it was used to identify and demonstrate the existence of isotopes, to determine their relative abundance and their exact atomic mass. Around the '40s and '50s mass spectrometry appeared in chemistry mainly with the analysis of organic molecules.

3.1. The mass spectrometer

The mass spectrometer is an instrument consisting of different parts shown in Figure 2. The analysis in mass spectrometry involves the following events:

- Introduction of the sample

- Ionization of sample molecules within the ion source

- Analysis of ions produced

- Ion detection/data analysis

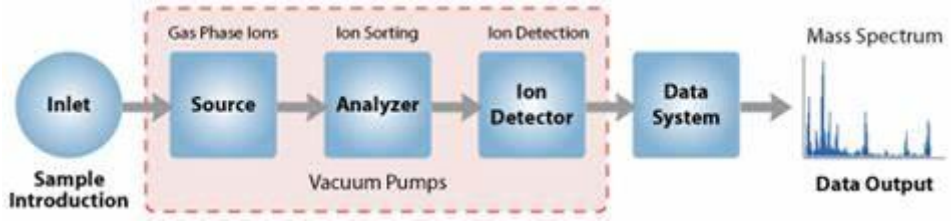


Fig. 2 Block diagram of a mass spectrometer, modified from premierbiosoft.com.

The sample, which can be solid, liquid, or gaseous, is introduced into an ionization source and, from here, the obtained ions are directed to the analyzer placed in the vacuum, necessary to ensure the free average path of the ions produced. Depending on the polarity of the sample being analyzed, the sample may already be in solution ion form (in this case, the ion source will provide gas-phase ion stabilization) or it may be ionized by several possible methods in the ion source. The ions produced, present in the gas phase, are separated in the analyzer based on their mass-to-charge ratio (m/z) and are collected by a detector in which they generate an electrical signal proportional to the number of ions present. The data processing system records these electrical signals as a function of the m/z ratio and converts them into a mass spectrum. For the analysis carried out in this work, it was decided to couple the HPLC to an ESI source, an electrospray type source, commonly used for the analysis of different samples in the HPLC techniques coupled to mass spectrometry. The main difference of this "soft" ion source respect to others, is its ability to ionise a molecule while minimising unwanted breakage of parts of the molecule.

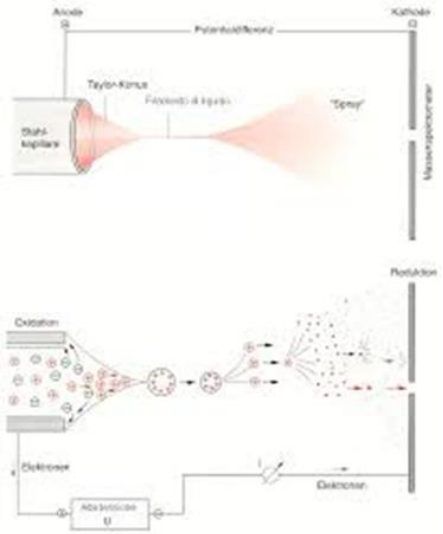


Fig. 3 Scheme of an Electrospray Source

A mass spectrum is a graph that represents the relative amounts of ions as a function of the mass to charge ratio. Since molecules are extremely small entities, Dalton (Da) is used as the unit of measurement for their molecular mass, where 1 Da is 1/12 of the mass of the ^{12}C isotope. The electric charge instead is a quantized property, which exists only for integer multiples of the elementary charge or that of the electron or proton, for this reason the charge of an ion is expressed as the number z of elementary charges. The mass to charge ratio is therefore expressed in terms of Da per elementary unit of charge. In mass spectrometry most ions have only one charge (z=1) so that their m/z value corresponds numerically with the molecular mass in Da. The ions and their relative intensity allow to establish the molecular weight and structure of the compound under examination. Since the ionization process can cause fragmentation of the original molecule, ions with a different m/z ratio than the one corresponding to the molecular weight of the sample often appear on the spectrum**.** A similar result can be obtained if a "tandem" approach (MS/MS) is used where the isolation of an ion is followed by its fragmentation to allow its qualitative characterization. During the analysis to ionize the sample we used the technique of electronebulization (electrospray- ESI), which is easily interfaced with HPLC and has the characteristic of being able to analyze ions with a high m/z ratio and therefore molecules with a molecular mass even higher than 200,000 Da. Consequently, the technique can be also used to determine the molecular weight of macromolecules such as proteins. The sample is dissolved in a solvent (methanol, water, acetonitrile, acetone) and then introduced into the ionization chamber, where it is nebulized by atmospheric pressurization through a needle held at high electrical potential and comes out in the form of aerosols. The charged particles that have formed are attracted by an ion

extraction lens, due to the electric field between it and the capillary needle. Due to their small size the solvent evaporates, and each droplet becomes smaller and smaller. The formation of analyte ions follows the mechanism of Coulombian fission, illustrated in Figure 3; initially the larger droplets become smaller by evaporation of the solvent, consequently there is a higher concentration of charges of the same sign in the particle; this increases the repulsions until they exceed the surface tension and determine the expulsion of smaller and smaller analyte ions from the droplet. Finally this process continues until individual ions are obtained which can be also gifted with more positive $[M+nH]^{n+}$ or negative $[M-nH]^{n-}$ charges. The ions are pushed through a slit system where they are accelerated to the analyzer. A peculiar characteristic of this ionization technique is the ability to generate multicharged ions, which allows to analyze substances with high molecular weight. As the number of charges (z) increases, the m/z ratio decreases, so very high mass substances can be analyzed. This is very advantageous for the analysis of high mass molecules such as proteins and other biomolecules, making the modifications or alterations contained therein detectable. In order to understand if we are dealing with a multi-charged ion, we have several possibilities but the simplest is to observe the difference in mass between the ion and the relative isotopic abundance of carbon contained in it. In a mass spectrum of an organic monocharged molecule it is easy to identify the relative isotopic abundance of carbon because it manifests itself on the spectrum as a peak that has a ratio m/z equal to the mass of the ion +1 (Carbon 12 and Carbon 13 differ by 1 uma). With reference to the m/z value we can therefore guess how for multicaricated ions this difference in mass unit between the molecular ion and its relative isotopic abundance of carbon will be 0.5 uma for a bi-charged ion, 0.33 uma for a tri-charged ion and so on.

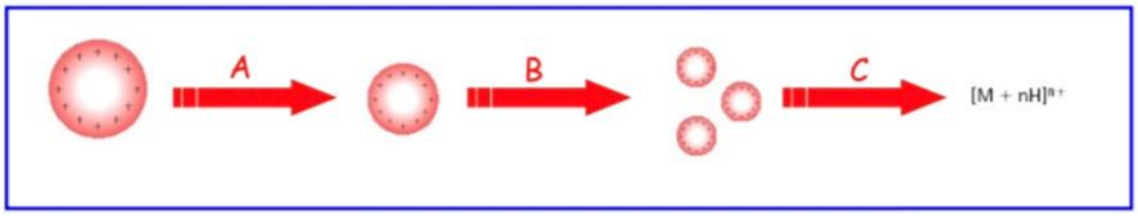


Fig. 4 Scheme of Coulumbian fission

3.2 Ion trap analyzer

The analyzer is the part of the instrument that selects and separates ions on the basis of their m/z ratio, therefore with entities of different masses, and reveals each charged molecule with a certain mass, sequentially over time. So, in the spectrum ions with different m/z ratio generate different peaks because these reach the detector at different times. During this work we used the ion trap analyzer, a component formed by three electrodes with hyperbolic inner surface that allow to retain the ions inside them. An alternating or radio frequency (RF) electric field is applied to the central electrode, a continuous electric field (DC) is applied to the two lateral electrodes; these generate a hyperbolic electric field capable of trapping ions with a certain m/z ratio. The ions oscillate between the two caps and the inside of the ring, remaining for a certain period of time (definable) within this space. There are two holes in the two side caps that allow the ions to enter from the source and to exit toward the detector. The mass spectrum is obtained by increasing the RF potential so that it increases the oscillation of the ions that are directed towards the detector and ejected sequentially according to their increasing m/z ratio.

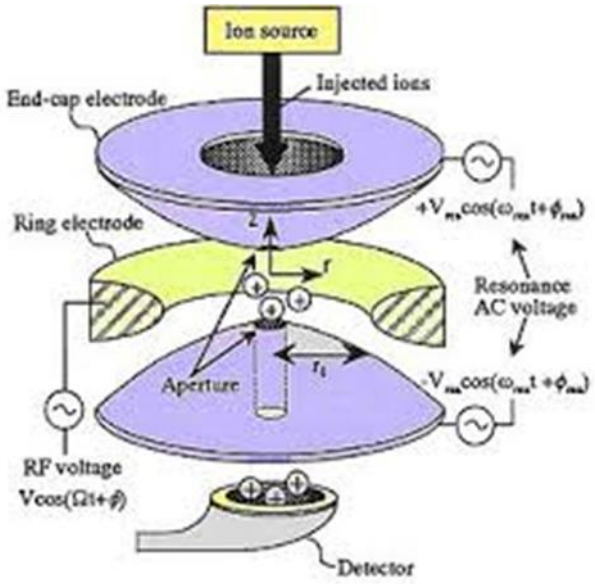


Fig. 5 Ion trap analyser. The separation takes place within the space between the electrode "covers" and the ring electrode.

4 EXTRACTION METHOD

The extraction of primary and secondary metabolites from a complex eukaryotic organism such as a fungus can be more or less difficult. Over the years, numerous protocols for the extraction of fungal molecules have been tried and tested, but they are unsatisfactory for this project for several reasons:

1- are protocols aimed at the extraction of metabolites produced by microorganisms cultivated on petri dish.

2- are protocols aimed at the extraction of a specific class of molecules omitting an overview of the metabolites produced by that particular fungal species.

3- are protocols that concern the total extraction of metabolites but produced by anamorphic fungi or yeasts that allow to have less interference problems related to the presence of chitin and/or other polysaccharides.

In our case, studying a complex organism that is a basidiomycete in its sexual life phase, these protocols are not applicable. The first extraction attempts that have been conducted concerned parts of pseudo-tissue of the fresh fungus placed in a vial containing the extraction solvent. Although we realize that this technique is very crude, it was necessary to have a first image of the work to be done. The use of the fresh mushroom has proved to be full of problems: the matrix is too complex, so there is no possibility of an efficient extraction of metabolites. To avoid a strong interference of the fungal cell wall, rich in chitin and other polysaccharides, it was decided to proceed to a separation of the different parts of the basidiomata in aliquots smaller than the weight of about two grams each. Subsequently, the sample thus separated was dried

into an Eppendorf. The drying process must be complete as the residual internal vegetation water of the fungal sample is likely to generate a phenomenon of revival of carbohydrates once they are inserted in the extractive medium. It was decided to proceed to an extraction of the different parts of the fungus that are from the point of view of the production of absolutely equivalent metabolites, since the fungus is not divided into real tissues but simply into pseudo tissues that, despite the different morphology, have the same function and do not have such a strong specialization as real animal tissue.

Fig. 6 Dried samples of *Pleurotus ostreatus*

This type of subdivision allows to have samples that are from the genetic and biochemical point of view extremely similar, if not even equal, thus reducing the error due to a differentiation or specialization or different expression of metabolic genetic characteristics as it happens for example in the tissues of animals or superior plants. Extractive solvents to obtain samples that can be analyzed through the technique of HPLC coupled to mass spectrometry are different. Having chosen to use an analytical technique that provides for the use of mobile phases water and acetonitrile with the

addition of formic acid and to have as broad a representation as possible of the different classes of metabolites contained within our sample, the extractive solvents were different. Ethanol was chosen as the main solvent and in the first experiments, to which chloroform had been coupled in parallel extractions, it gave good results.

Fig. 7 Powdered samples of *Pleurotus ostreatus* before extraction

The main problem with chloroform is that the extracts obtained with this solvent must be brought, after extraction, to a very high dryness because chloroform itself is incompatible with the most common stationary phases of the chromatographic columns used, in our case an Atlantis C18 column. For this reason, not to be understated because the optimal preparation of the sample is essential to have good results with these analytical techniques, the use of chloroform has been soon abandoned. It was therefore decided to associate the extraction in ethanol with the parallel extraction of samples from the same fungus with other solvents as methanol and acetonitrile.

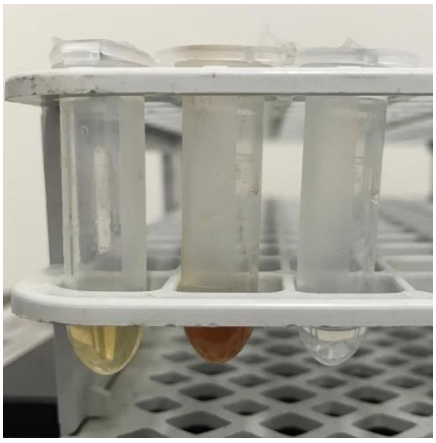


Fig. 8 Comparison between the three different partially dried extracts; from left to right ethanolic, methanolic and acetonitrilic extracts.

These three solvents have polarities, quite similar for ethanol and methanol, but different for acetonitrile which is the most apolar of the three. Another very important point concerning the extraction was the choice to reduce our sample to powder in mortar; the reduction in fine or semi-fine powder allows to increase considerably the contact surface of the sample with the extracting solvent increasing the extraction efficiency and the amount of dry extract obtained. In the first attempts, starting from a fresh sample of about two grams, dry extracts of about 1.5 to 2 milligrams were obtained. Although apparently this is more than a sufficient amount to obtain good HPLC full scan and tandem mass analysis, it must be remembered that this is not an extract containing a pure molecule or few molecules, but an extremely complex raw extract. Moreover, the extraction inevitably leads to the solubilization of chitin oligomers or other polysaccharides associated with the fungal wall. The presence of these polysaccharides affects the analytical separation carried out. Indeed, polysaccharides have a considerable capacity to form hydrogen bonds with organic molecules, thus creating what we could call a sponge effect, retaining the extracted

molecules and preventing them from being adsorbed by the column and then, in due time, dragging them away, masking their presence and preventing both the formation of ions and the detection by the mass spectrometer. To overcome this problem, it was decided to proceed with hot extraction at 65°C for two hours followed by the rapid cooling at 4°C to get the precipitation of most carbohydrates. The extract was filtered on filter paper and centrifuged for 15 minutes at 13,000 rpm. Once these operations have been completed, the residue of chitin and other polysaccharides were arranged on the surface of the extract in the form of flakes that can be easily removed mechanically using a small spatula. Before the HPLC-MS analysis, the layer was resuspended in 1 mL of a milli-q water: acetonitrile solution 50:50. A simple comparison of the mass of the extracts brought to dryness highlights the differences in the production of molecules in the two strains of *Pleurotus ostreatus* used and how the substrate enriched with lavender is a stimulus that leads to an increase in the production of primary and secondary metabolites.

5 HPLC- MS and MS-MS TECHNIQUES

High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) is a powerful analytical technique, useful to obtain a wide range of information regarding molecules, such as to identify unknown products, have qualitative and quantitative determination of known molecules and clarify structure and chemical properties of many different substances. Its use for complex matrices is well documented [22; 23] and the introduction of both an electrospray ion source for ionization and tandem mass analysis has revolutionized the research on metabolites and other chemicals [24]. Two major advantages of an ESI source's use compared to other ion sources are its capabilities of adding or subtracting one or more protons to/from a molecule and, at the same time, the avoidance of derivatization during sample preparation and undesired fragmentations during sample analysis, thus allowing this technique to be useful both for structural characterization and quantitative determinations of compounds. Fundamental is the availability of softwares to analyze mass spectra, enabling, moreover, the isolation of a single ions' family from the total ion current. All these features make the electrospray ionization source probably the most used technique for ionization of polar molecules, ranging from low to really high molecular weights. Fungi are an interesting, increasingly studied and ever-growing source of new molecules with potential benefits on human health [25; 26]. *Pleurotus* mushrooms are known as incredible providers of different nutrients and molecules with known beneficial effects such as vitamins, aminacids, essential fatty acids and their nutritional value is well known [27; 28] and at the same time numerous properties are known such as anti-inflammatory, stimulant and antioxidant properties possessed by the wide pattern of primary and secondary

metabolites produced by it [29; 30; 31]. However, the study of their metabolites and their potential is usually carried out without characterization of their structure and concerning only certain aspects. The available literature focuses on works that use long-known essays which, while providing the necessary indications on the type of activity of the molecules, on their reducing power; they do not indicate the real structure of the molecules involved [32; 33]. The greatest advantage of the HPLC-MS approach lies in the possibility to complete scan of the entire metabolomic pattern produced by the model organism, simplifying, and speeding up the analyses aimed at characterizing and quantifying molecules of interest.

5.1 Preliminary analysis method

For the creation of a mass analysis method that would be satisfactory for a sample as complex as a raw fungal extract, several literature works have been consulted. All these works, however, were unsatisfactory; applying different literature protocols we obtained non-optimal separations of the different components extracted from the fungus, in particular of the polar compounds for which the obtained signals were poorly resolved with, as consequence, a poor isolation and not-optimal fragmentation of ions by the mass spectrometer. In fact, to better separate the polar fraction of the extracts, the first chromatographic separations were carried out in isocratic mode. When residues of chitin and other polysaccharides of the wall were present, since this was still inevitable, despite the development of an optimized extraction method, these compounds were dragged inside the column with the result that all these polysaccharide residues create a deficit of ionization at the level of the ESI source and a disturbance for the detectors, both HPLC and the mass spectrometer. In the first

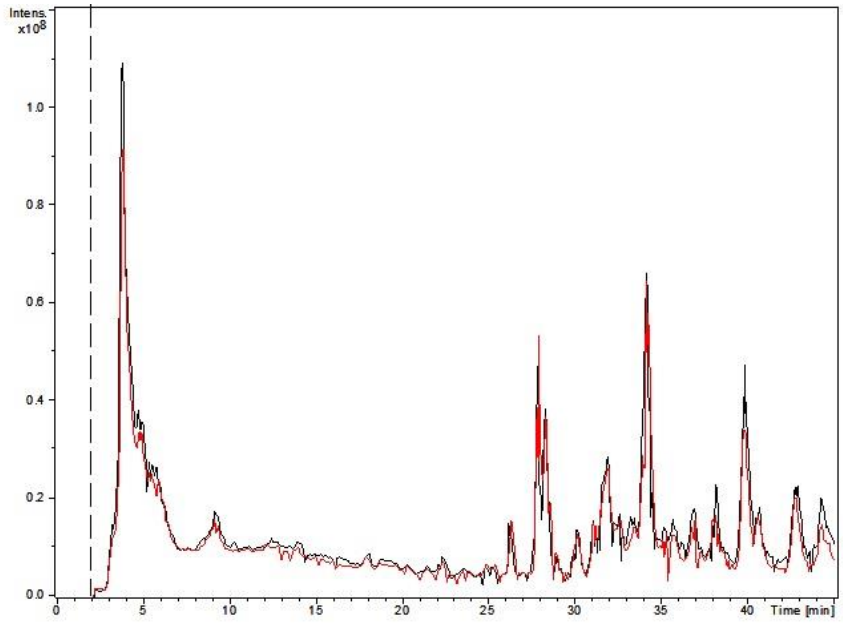
analysis in fact, it could be noted that all polar molecules were simply detected by the chromatograph and mass spectrometer as a single huge wide peak present in the first minutes of analysis. To overcome this problem, we chose to proceed with a direct linear gradient that allowed an outflow of the polysaccharides in the early minutes of the chromatographic run, allowing the retention of many molecules of interest and minimizing the risk of column clogging and problems in ionization at the level of the ESI ion source. Therefore, despite an apparent loss of information in the polar fraction of the extract, it was possible to identify certain molecules in it at a qualitative level.

5.2 Sample preparation and liquid chromatography

Samples were prepared by the use of a Speed-Vac system; each sample (2 g fresh weight) was placed in a 2 mL Eppendorf tube and dried. After drying, each sample was weighed. For extraction, the dried sample was pulverized in a mortar and the obtained powder was placed in a glass flask. Anhydrous ethanol (2 mL) was then added to all powdered sample. The sample was left on a plate heated at 55°C for two hours, under stirring using a magnetic stir bar. The extract was filtered using filter paper and then centrifuged for 10 minutes at 13,000 rpm. Centrifugation is a crucial point of the sample preparation: as already mentioned, the extraction depends on the removal of chitin oligomers still present in the sample which interfere with the analysis in HPLC-MS both sequestering molecules from the medium with a "sponge effect" and interfering with the optimal ionization of the metabolites in the ESI ion source. The HPLC-ESI-MS analysis has been performed using an Agilent 1100 chromatograph directly coupled with an MSD ion trap mass spectrometer. Chromatographic separation was conducted using a C-18 Symmetry column (Waters).

The choice of the column has been made considering the complexity of the matrix to be analyzed and the reproducibility of the method. Chromatographic separation was carried out using a linear gradient of water and acetonitrile in reversed phase, going from 100% water to 100% acetonitrile in 40 minutes, keeping 100% acetonitrile for 5 minutes, followed by 20 minutes of post-run to reach the initial conditions; the flow was 0.3 mL/minute with the eluent directly sent to the MS interface in the ESI source. Temperature was set at 25°C. Before injection, each sample was taken in water and acetonitrile 50:50, sonicated for 15 minutes, centrifuged, and then transferred in an injection vial. The full scan analysis has been performed selecting a m/z mass range between 50 and 800 AMU, which is a good window to detect molecules of different nature produced by the fungus.

A



B

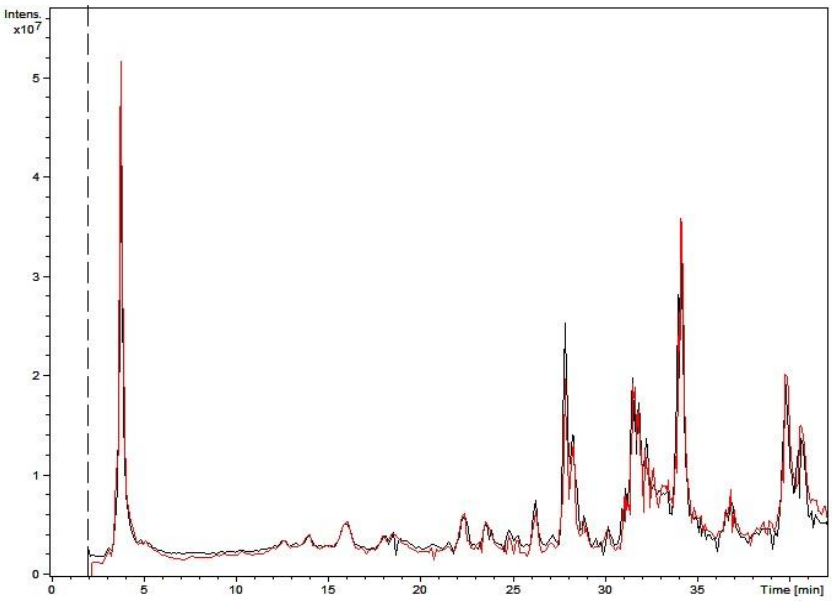


Fig. 9 Comparison between two chromatographic separation of different samples of the same strain, grown under the same conditions and extracted in ethanol (A) and acetonitrile (B), the perfect overlap of the different extracts guarantees reliability and reproducibility of the extraction and analysis method.

5.3 Identification of compounds by DATA BANKS querying

The Massbank EU database was started as a public data repository in Japan in 2006 and contains many spectra, obtained from different types of analysers (triple quadrupole, ion trap, ion-trap, q-TOF, Q-TRAP, etc.) and spectra (full scan and/or tandem). It belongs to the NORMAN network which includes reference laboratories, research centres and similar organizations for monitoring emerging environmental substances. NORMAN joined Massbank in 2012 and founded Massbank EU. This database includes, up to today, 46,334 spectra for metabolites obtained from 32 contributing institutions. To start a search, the m/z ratio of the parent ion is entered and the m/z ratios of one or more fragment ions can be entered. A relative intensity of 100 and an adequate tolerance to the accuracy and resolution of the instrument used is set. Although the used instrument has, for the considered m/z ratios, an accuracy of 0.05 UMA, a tolerance of 0.3 UMA was set. The chromatograms and the relative spectra were at first analysed through a data analysis software supplied with the instrument. As an example, the complete scan and the tandem mass spectra obtained for linoleic acid are reported, as well as the identification process is briefly described. Starting from the total ionic current, the instrument selected, isolated, and fragmented an ion with the m/z ratio equal to 279.4 (parent ion).

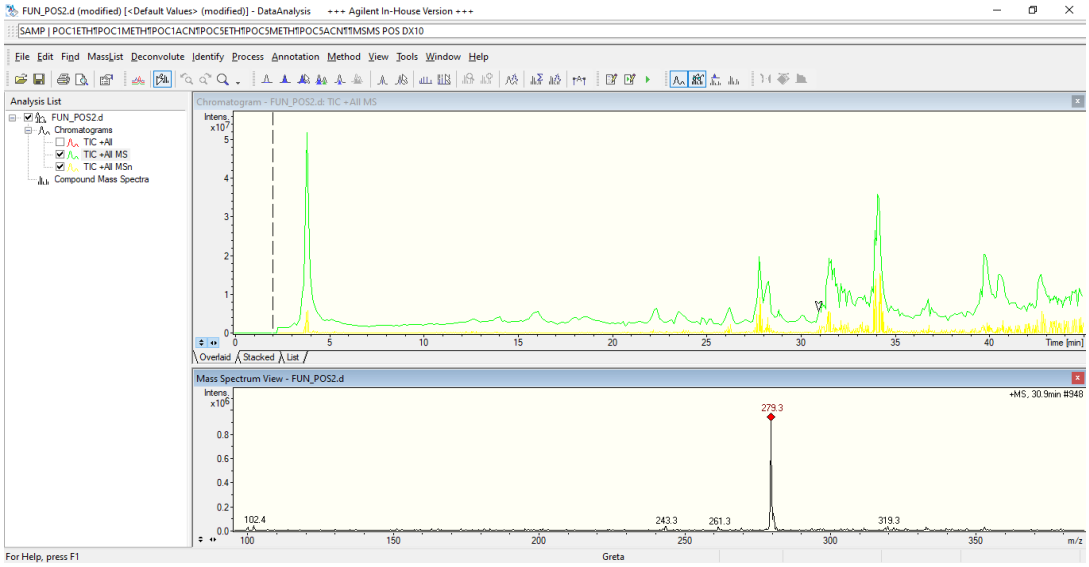


Fig. 10 Representation of the total ion current and isolation of the ion m/z = 279.4 from the analysis of a *Pleurotus* extract

The spectrum obtained by the fragmentation of the parent ion (MS/MS spectrum) was selected.

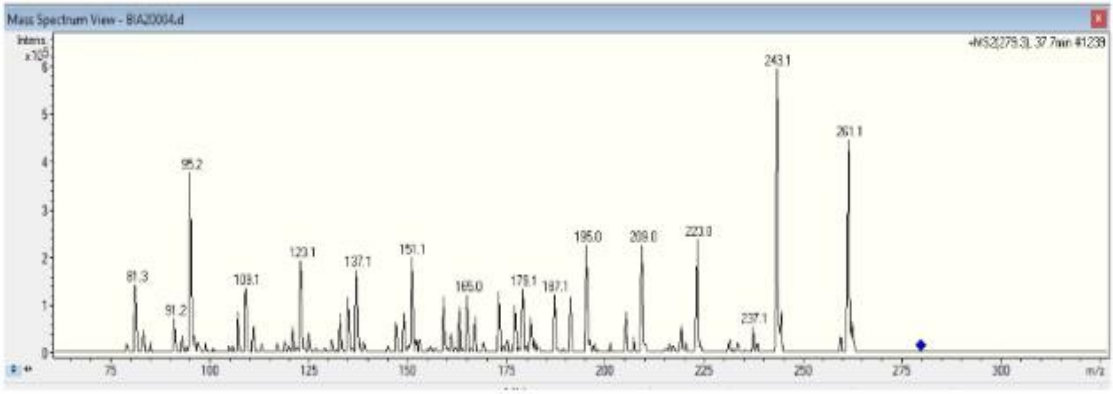


Fig. 11 MS / MS spectrum of ion 279.4

To proceed with the online interface of MassBank, the m/z values of the parent ion and of the most relevant ions (up to 10 m/z values) were inserted in the dialog window and then the tool was able to proceed with a comparative analysis of the values entered

with the full scan and fragmentation spectra present on MassBank. In this specific case, since there was correspondence between the peaks of the spectrum of linolenic acid present in the database with that obtained experimentally, it could reasonably be concluded that the substance under consideration is linolenic acid. For the certain identification of the molecules under study, the results obtained by comparing the experimentally acquired spectra and those present in the database can be validated through the use of pure standards. This means that the univocal attribution of a given signal to a specific molecule must go through the comparison of the MS and MS/MS spectra obtained from the analysis of the sample and from that of the commercial standard.

5.4 Reagents and chemicals

We used, as mobile phase, HPLC grade acetonitrile (Merck, Darmstadt, Germany) and Milli-Q water (Millipore Corp., Bedford, Italy). Both have been filtered, degassed and an addition of respectively 0.5% and 1% formic acid (Carlo Erba reagents, Italy) was done to facilitate and improve ionization. SIAD (Bergamo, Italy) provided nitrogen at research grade (> 99.995%).

5.5 Results

In the tables below (paragraphs 5.4.1-4) the putative molecules found with the adopted analytical method are reported. The tables were divided by *Pleurotus ostreatus* strain, positive or negative ion mode and cultivation substrate. All the analysed spectra are stored at the mass spectrometry laboratory of the Centre of Excellence for Biomedical Research (CEBR) in Genoa. In each table the retention time of each compound (RT), the parent ion and its fragments are reported.

5.5.1 Molecules from *Pleurotus ostreatus* cultivated on standard substrate, positive ion mode

RT (min)	Putative molecule	Parent ion	Fragment	Fragment	Fragment	Fragment	Fragment
2.3	Dimethylglicine	104	86				
2.4	L-Carnitin	162	103	85			
3	Methilguanine	166	149	130	106	105	73
3.2	Panthotenic acid	220	148	90			
3.2	3-hydroxy-C14 homoserine lactone	328	310	292			
3.4	Dehydrocostus lactone	231	213	203	185	175	157
3.7	N-fructolsil isoleucine	294	276	258	248	230	144
4	Adenosine	268	136				
4.1	3-Indolebutyric acid	204.5	186.1	168	158.1		
4.2	Dihydrocaffeic acid	181	137				
4.3	N-Acetylmuramic acid - H2O	294.4	276.2	210	186		
4.5	Acetyl-N-Carnitin	204	145	85			
4.7	Nicotinic acid	124					

5.4	Uridine 5'-monophosphate	323	211				
5.6	Lactaminic acid	308	170	98	87		
5.8	Thyrosine	182	165	147			
5.9	Tryptophanamide	204	132				
6.7	4'-hydroxyflavanone	241	219				
8.5	Threose	121	93	91	77		
9.2	Inosine	269	137				
9.3	Guanosine	284	152				
9.4	Hydro-epicatechin	310	292	274			
9.5	Hydro-phenylalanin	178	166	120			
9.8	5'-S-Methylthioadenosine	298.8	136				
10.8	Triptofane	205	188				
11	Trehalose	203	185				
11.1	Galactinol	341	202	179	161		
11.1	Sebacic acid	201	183	139	111		
11.4	Altenuene	293.3	275	257			

11.5	Jasmonic acid	211	193	151	133		
11.7	N-Fructosyl isoleucine	294.3	276	258	230	212	
12.4	Resveratrol	229	211	194			
12.7	Sinapic acid	225	207				
13.1	Cathechin -like	291	273	249	207		
13.2	Biotin	245.2	227	199			
13.,5	3,4,5'-Trihydroxy-3'-glucopyranosylstilbene	407	245				
14	Stearic acid	283					
14	N-(n-octyl)-2-pyrrolidinone	198	86				
14.2	Cytidine	244	198				
14.7	N-Fructosyl phenylalanine	328	310	292	264	97	
15.2	Simil Flavon spectrum	329	302	285			
15.3	N-Fructosyl pyroglutamate	290	200	128.2			

15.6	Hexanoyl-L-Carnitine	260	201	99	85		
15.7	Riboflavin	377	243	198	172		
16.2	L(+)-Cystathionine	223	177	149			
16.5	N-Acetylphenylalanine	208	190	162	120		
18.4	Cytidin	244	198	86			
18.8	C10-homoserine lactone	256	238	228			
19.9	Glycolithocholic acid	434.5	359.5				
20	Glutathione	307	288	272	254	210	179
20.3	Undecenoic acid + Na	195	183				
21.4	Sphingosine + H2O	318	300	282			
22.6	N-Fructosyl gamma-glutamyl-S-methylcysteine						
23.1	Tetrahydrobiopterin + phenylalanin	431.3	265.8				

23.8	6-Hydroxy-4-methylcoumarin	177	149				
24.4	Linolenic acid	279	261	243			
24.6	Gibberrelin like	349	329	303	284		
25.5	1-(3-carboxypropyl)-3,7-dimethylxanthine	267	249	221			
25.6	Fisetin	287	259	163.8	147.4		
30	Linoleic acid	281	265	245			
30.1	Hydroheptilidic-acid	299.3	281.1	265	245		

5.5.2 Molecules from *Pleurotus ostreatus* cultivated on enriched substrate, positive ion mode

RT (min)	Putative molecule	Parent ion	Fragment	Fragment	Fragment	Fragment	Fragment
2.3	Dimethylglicine	104	86				
2.4	L-Carnitin	162	103	85			
3	Methilguanine	166	149	130	106	105	73
3.2	Panthotenic acid	220	148	90			
3.2	3-hydroxy-C14 homoserine lactone	328	310	292			
3.4	Dehydrocostus lactone	231	213	203	185	175	157
3.7	N-fructolsil isoleucine	294	276	258	248	230	144
4	Adenosine	268	136				
4.1	3-Indolebutyric acid	204.5	186.1	168	158.1		
4.2	Dihydrocaffeic acid	181	137				
4.3	N-Acetylmuramic acid - H2O	294.4	276.2	210	186		
4.5	Acetyl-N-Carnitin	204	145	85			
4.7	Nicotinic acid	124					
5.4	Uridine 5'-monophosphate	323	211				

5.6	Lactaminic acid	308	170	98	87		
5.8	Thyrosine	182	165	147			
5.9	Tryptophanamide	204	132				
6.7	4'-hydroxyflavanone	241	219				
7.5	5-Cholanic acid 3-OL-6_7-dione	351.3	333	315			
8.5	Threose	121	93	91	77		
9.2	Inosine	269	137				
9.3	Guanosine	284	152				
9.4	Hydro-epicatechin	310	292	274			
9.5	Hydro-phenylalanin	178	166	120			
9.8	5'-S-Methylthioadenosin	298.8	136				
10.8	Triptofane	205	188				
11	Trehalose	203	185				
11.1	Galactinol	341	202	179	161		
11.1	Sebacic acid	201	183	139	111		
11.4	Altenuene	293.3	275	257			

11.5	Jasmonic acid	211	193	151	133		
11.7	N-Fructosyl isoleucine	294.3	276	258	230	212	
12	3-Hydroxy-7 Ketolithocholicacid	391.3	373	355	337.1		
12.4	Resveratrol	229	211	194			
12.7	Sinapic acid	225	207				
13.1	Cathechin -like	291	273	249	207		
13.2	Biotin	245,2	227	199			
13.5	3,4,5'-Trihydroxy-3'-	405	243				
14	Stearic acid	283					
14	N-(n-octyl)-2-pyrrolidinone	198	86				
14.2	Cytidine	244	198				
14.7	N-Fructosyl phenylalanine	328	310	292	264	97	
15.2	Simil Flavon spectrum	329	302	285			
15.3	N-Fructosyl pyroglutamate	290	200	128.2			
15.6	Hexanoyl-L-Carnitine	260	201	99	85		
15.7	Riboflavin	377	243	198	172		

16.2	L(+)-Cystathionine	223	177	149			
16.5	N-Acetylphenylalanin	208	190	162	120		
17.3	13-KODE	293	275	249	236	221	
17.5	9-HPODE	311	293	275	249		
18.4	Cytidin	244	198	86			
18.8	C10-homoserine lactone	256	238	228			
19.9	Glycolithocholic acid	434.5	359.5				
20	Glutathione	307	288	272	254	210	179
20.3	Undecenoic acid + Na	195	183				
20.5	9,10-DiHOME	313	295	277	201		
21.4	Sphingosine + H2O	318	300	282			
23.1	Tetrahydrobiopterin + phenylalanin	433.3	267.8	165.8	149.4		
23.7	12,13-EODE	295	277	233	195		
23.8	6-Hydroxy-4-methylcoumarin	177	149				
24.4	Linolenic acid	279	261	243			
24.6	Gibberrelin like	349	329	303	284		

25.5	1-(3-carboxypropyl)-3,7-	267	249	221			
25.6	Fisetin	287	259	163.8	147.4		
26.6	13-HPODE	311	293	249			
30	Linoleic acid	281	265	245			
30.1	Hydroheptilidic-acid	299.3	281.1	265	245		
32.4	13-HpOTrE	309.4	291	275			
35.4	Cholanic acid	375.3	357.2	339.3	321.3		
37.4	12,13-EODE	295	277	251	233	195	183

5.5.3 Molecules from *Pleurotus ostreatus* cultivated on standard substrate, negative ion mode

RT (min)	Putative molecule	Parent ion	Fragment	Fragment	Fragment	Fragment	Fragment
3.2	Pantothenic acid	218.1	146	88			
3.4	Galactinol	341	202	179	161		
3.6	D-Glucose 6-phosphate	259	199	169	139	97	79
5.4	Uridine-5-monphosphate	323	211				
5.9	Tryptophanamide	202	130				
6.3	Uridine	243	200	182	153		
7.3	4-Hydroxy-L-proline	307	113	84			
9.3	Guanosine	282	150				
10	Adenylosuccinic acid	462.2	444	418	400	3364	266
11.1	Sebacic acid	201	183	139	111		
12.2	Phosphatidylcholine	536	256				
13.5	3,4,5'-Trihydroxy-3'-	405	243				
14	Stearic acid	283	265	243			
16.3	N-Acetylphenilalani	206.2	164	147			

17.1	Myristoleic acid	225	207	199	185	171	
17.3	Gibberellin like	345	327	309	300	227	
17.5	Hydroxyoctadecenoic acid	311.3	293	275	261		
20	L-Glutathione	307	288	272	254	210	179
20.3	Undecenoic acid + Na	195	183	165	147	134	
21.1	Jasmonic acid	209	165				
21.3	Dihydrocaffeic acid	181	137				
23.1	Tetrahydrobiopterin + phenylalanin	431.3	265.8	163.8	147.4		

5.5.4 Molecules from *Pleurotus ostreatus* cultivated on enriched substrate, negative ion mode

RT (min)	Putative molecule	Parent ion	Fragment	Fragment	Fragment	Fragment	Fragment
3.2	Pantothenic acid	218.1	146	88			
3.4	Galactinol	341	202	179	161		
3.6	D-Glucose 6-phosphate	259	199	169	139	97	79
5.4	Uridine-5-monphosphate	323	211				
5.9	Tryptophanamide	202	130				
6.3	Uridine	243	200	182	153		
7.3	4-Hydroxy-L-proline	307	113	84			
9.3	Guanosine	282	150				
10	Adenylosuccinic acid	462.2	444	418	400	3364	266
11.1	Sebacic acid	201	183	139	111		
12.2	Phosphatidylcholine	536	256				
13.5	3,4,5'-Trihydroxy-3'-	405	243				
14	Stearic acid	283	265	243			
14.6	13-HPODE + H2O	329	311	293			

16.3	N-Acetylphenilalani	206.2	164	147			
17.1	Myristoleic acid	225	207	199	185	171	
17.3	Gibberellin like	345	327	309	300	227	
17.5	Hydroxyoctadecenoic acid	311.3	293	275	261		
17.5	9-HPODE	311	293	284	275	249	
17.7	13-KODE	293	275	249	236	221	
20	L-Glutathione	307	288	272	254	210	179
20.3	Undecenoic acid + Na	195	183	165	147	134	
20.5	9,10-DiHOME	313	295	277	201		
21.1	Jasmonic acid	209	165				
21.3	Dihydrocaffeic acid	181	137				
23.1	Tetrahydrobiopterin + phenylalanin	431.3	265.8	163.8	147.4		
23.7	12,13-EODE	295	277	201			
26.8	9-HODE	295	277	171			
31	13-HPODE	311	193	249			

5.6 MOLECULES FOUND AND THEIR FUNCTION

Analyses showed that cultivated mushrooms are an incredible source of various molecules with potential benefits for human health. Specifically, the comparison between molecules derived from different strains revealed that the wild strain is a better producer of secondary metabolites in response to the presence of environmental stressors. This greater and more varied production is probably due to the fact that the domestic strain, cultivated for generations in a protected and controlled environment, through cloning of the mycelium, i.e., an asexual type of reproduction that does not allow genetic mixing, depresses or silences over the generations various secondary metabolic pathways that are no longer useful for maintaining physiological activity. These metabolic pathways are not silenced in the wild strain, as in nature it is in direct contact with environmental stressors, direct competitors, and pathogens. The substrate enriched with lavender, a plant particularly rich in terpenes and other molecules produced by it as antibacterial and antifungal agents, stimulated the fungus to activate its secondary metabolism, as evidenced by the presence of numerous molecules that are not part of the fungus' usual primary metabolomic pattern. Although weighing data from the different growth spurts of the fungus showed that fungus grown on straw generally gave better results in terms of biomass, HPLC-MS and tandem mass analysis revealed that the levels of n-acetylglucosamine and its precursors remained constant in each sample. This indicates that there is no direct correlation between the action of the lavender-enriched growth medium and the lower biomass yield of the fungus grown on it, as the constituents of the cousin wall that dictate less the growth and hyphal elongation of the mycelium remain constant. The molecules, isolated and characterised from the fungal samples, were several and belong to numerous classes

of chemical compounds. The main families of isolated chemical compounds will be analysed below, as well as their function within the fungal organism and their possible use in products intended for human health will be briefly described. In particular, it will be shown that the fatty acid epoxides, particularly myristoleic acid, linoleic acid and linolenic acid are produced by the fungus grown on lavender-enriched substrate.

5.6.1 Nitrogenous basis and nucleosides

Nucleotides are endogenous compounds that are of primary importance in the synthesis of nucleic acids, cell signalling, enzymatic regulation, and metabolism. All the main nucleosides and all the main purine and pyrimidine nitrogen bases were identified in the analysed samples. The high concentration of cyclic nucleotides, in particular of cyclic AMP and cyclic GMP, is of great interest. Cyclic nucleotides are considered among the main secondary messengers in many physiological signal transduction reactions. Fungi are known to produce nucleotides and nucleosides of different nature, and in particular several cyclic nucleotides produced seem to have important roles in suppressing tumour function. Some synthetic derivatives of these compounds have been investigated and show to have anti-cancer action. However, the antitumour properties of nucleotides and the activity of *Pleurotus ostreatus* products have not been analysed yet. The analysed samples from *Pleurotus ostreatus* did not show any particular differences in the production of nitrogenous bases, nucleosides, in relation to the growth substrate. It would be important and interesting to further investigate the role these molecules play in the regulation of cell growth both in the fungus and ex vivo with mammalian cells [34].

5.6.2 Amino acids

From the analysed samples, a good amino acid component emerged. In particular, several essential amino acids for adult humans, are present, specifically phenylalanine, tryptophan, lysine, leucine and isoleucine, or their fructose or ribose glycosylated derivatives. In addition to the undoubted nutritional and nutraceutical value of these amino acids, it is interesting to note that glycosylated amino acids are molecules with known immunomodulatory and immunostimulant functions. These functions, carried out by these small molecules, should be studied in greater depth, since a role was discovered in the modulation of various pathological processes such as inflammation, and given their simple synthesis in the laboratory, large quantities of molecules with effective pharmacological action, low toxicity and high bioavailability could be obtained.

5.6.3 Dipeptides and tripeptides

Among the many molecules extracted and characterized, the amino acids glucosides and small dipeptides may be of great interest. Particularly known in the kingdom of fungi is the formation of cyclic non-ribosomal peptides, i.e. all those molecules that are produced by a mechanism of activation, bonding, cyclization. Example of these molecules are the penicillins that have been isolated for the first time from an anamorphic fungus of the genus penicillium. This tendency of fungi to produce molecules of non-ribosomal peptide origin seems to be well established and widespread within the kingdom. In addition to the above-mentioned example of penicillins, there are evidence that in the upper fungi there is an evolutionary tendency to form molecules of peptide origin; our extraction and characterization of different

glycosylated peptides dipeptides from *Pleurotus ostreatus* seems to confirm this data. The role of these peptides can be very different. It has been shown that these molecules have antifungal, antimicrobial and immunostimulant actions. Moreover, they contribute to growth and it seems that this is the ultimate reason why root fungus produces them. Evolution of these peptides can be seen in the large, cyclized peptides molecules produced by some fungi such as amatoxins of the Amanita genus, demonstrating a clear evolutionary trend that leads from simple molecules to complex peptide cycles with defensive functions. It is no coincidence that amanitine evolved toxins in general are among the most effective toxins produced by superior fungi with a clear defensive role. Therefore, the production of peptides and dipeptides in *Pleurotus ostreatus* seems more than ever archaic or at least less evolved, and it is no coincidence that the action of these molecules evaluated experimentally in vitro has proven to be effective in limiting cell growth or regulating the immune response, but in a highly non-specific manner [35]. Of great interest is the good presence of glutathione in the samples. Glutathione, a tripeptide consisting of cysteine and glycine linked by a peptide bond and glutamate linked to cysteine by an iso-peptidic bond, is a molecule with powerful and well-known antioxidant properties. Its action is highly relevant to free radicals and peroxide ions and largely justifies the powerful antioxidant action of *Pleurotus ostreatus* extracts analysed on cell cultures treated with oxidising agents.

5.6.4 Unsaturated fatty acids and their epoxides

Fungi are an excellent source of polyunsaturated fatty acids, whose role has long been recognized in the prevention of inflammatory heart disease and have other important biological roles. It is no coincidence that a deficiency in the diet of polyunsaturated fatty acids, in particular linoleic acid, has been shown to be directly related to deficits in the formation of the brain. Many are the roles that polyunsaturated fatty acids take on for man; the main ones that we can list can be summarized in:

Energy reserve

Energy source

Structural

Metabolic.

It is therefore evident that a balanced intake of polyunsaturated fatty acids is fundamental for human health. From the analyses carried out, the most represented fatty acids in *Pleurotus* fungi are oleic acid, myristoleic acid, linoleic acid, linolenic acid. We also have a good share of palmitic acid, which is however a saturated fatty acid. The samples of *Pleurotus ostreatus* grown on substrate enriched with lavender have also shown the presence of numerous molecules derived from the fatty acid, in particular a series of fatty acid deposits. These molecules are produced by the fungus in response to environmental stress, through the activation of the monoxygenase domain of cytochrome p 450, which is common to all fungi and highly preserved in evolution. In the analyzed samples, it is evident that the fatty acid epoxides present are directly derived from the fatty acids of the fungus itself, in particular from linoleic acid

and linolenic acid. In the samples cultivated on a layer enriched with lavender we noticed a partial decrease in the content of polyunsaturated fatty acids in favour of the presence of fatty acid epoxides. This trend is also confirmed by the presence, in the samples of fungi cultivated on substrate enriched with lavender, of colic acids, molecules of external origin, completely similar to those found in human bile stalls and mammals in general. These are molecules that are produced as they act as mobilization of fatty acids inside the fungal cells, pointing out that the fatty acids inside the fungal cell are not stored in prepared systems but stored simply in the form of lipid drops. The presence of fatty acid epoxides in fungi is very interesting. It is known in literature that fatty acids often have cytotoxic activity or possess other harmful effects on health. It is thus important to investigate their role for human health, given their presence in our fungi. However, the analyses conducted on cells' cultures, have revealed that the fractions richer in fatty acids and their epoxides, i.e., the extracts in acetonitrile, do not have a cytotoxicity significantly higher than that of other extracts. Fatty acids' epoxides are known to be produced by cells undergoing great stress as result of the action of the monooxygenase activity of the Cytochrome P450, common to all fungi [36]. It should be noted that epoxides of fatty acids are only present in fungi cultivated on enriched substrates and absent, or present in traces, in fungi grown on only straw. This could suggest the role of fatty acids epoxides as biomarkers of stress in Basidiomycota. In contrast to plants, where the role of peroxygenases in the production of fatty acid epoxides is known [37], in fungi it appears that only the monooxygenase domain of Cytochrome P450 is involved in their synthesis. Fatty acid epoxides are molecules with interesting and powerful biological actions. Their role in the regulation and suppression of inflammatory processes is

recognised [38]. However, several toxic and tumour growth-promoting activities are known [41], so further investigation to verify the activity of fatty acid epoxides produced by *Pleurotus ostreatus* would be useful.

5.6.5 Vitamins

Numerous vitamins have been extracted and isolated from the analysed *Pleurotus ostreatus* samples. In particular, the presence of several B vitamins, thiamine, riboflavin, biotin and pantothenic acid, has been already reported [42]. There are no particular differences in the production of these vitamins by the domestic strain compared to the wild strain and, with the sole exception of pantothenic acid which is slightly more present in the samples grown on layers enriched with lavender, there were no particular differences in the distribution of the presence of these vitamins in the various samples. The presence of these compounds, which function as precursors of coenzymes and exert other beneficial functions for human health, is of great importance as it confirms that *Pleurotus ostreatus* represents an excellent supplier of molecules useful for human health, particularly with nutraceutical and antioxidant action.

5.6.6 Lactones

Lactones are naturally occurring cyclic esters with a cellular signalling function, second messenger molecules and antioxidant molecules. The presence of serine- and homo-serine-derived lactones in the samples analysed suggests that these molecules are produced by the fungus as agents mediating hyphal elongation. This would indicate that cultivation of the fungus on lavender-enriched substrates, although stressful for

the fungus itself, does not inhibit cell growth. The ex vivo physiological function of these lactones has yet to be investigated.

5.6.7 Melanins

Melanins are secondary metabolites characterized by a heterogeneous complex of polymers of phenolic compounds. In higher fungi the complexity of melanins is very wide. These pigments contain covalent links between aromatic rings and chitin derivatives such as n-acetylglucosamine or glyceride membranes. This complexity of the molecules makes them apparently difficult to solubilize and indeed they have been rarely detected in our extracts. The localization of melanins inside the fungal cell may vary but usually they are in close contact with the membrane and with the fungal wall when the primary role is to protect them from external agents, in particular from oxidative stress induced by environmental agents. Melanins are also particularly present inside the fungal spores that represent the form of dispersion of the fungus, being the way through which the higher fungi reproduce sexually. These structures are extremely fragile and must be protected. Although it is reported in the literature that some fungi are able to synthesize different melanins in response to different environmental stress [43], we have not found this in *Pleurotus ostreatus*. In this fungus the main melanin and pheomelanin appears to be produced by a detoxification mechanism that is responsible for the polymerization of phenolic compounds. However, through our HPLC-MS analysis, we obtained evidence of eumelanin production in *Pleurotus ostreatus*. In particular, its precursors have been detected, specifically dopa, gamma glutamine and traces of lignans. This limitation in B melanin production in higher fungi seems to be a preserved feature, since data in the literature

show that in anamorphic fungi there is a larger variety of melanins. Among the main roles that melanins play in the fungi are certainly those of protectors from outer stressors, thus protecting the integrity of the membrane of the mushroom ifa. Another function likely exerted by melanins is acting as resistance agents against either pathogens or competitors or adverse characteristics of the growing environment. However, the extraction method chosen for these analyses did not allow an in-depth analysis of the melanins produced by *Pleurotus ostreatus*. In addition, since they are complex molecules with many hetero atomic rings condensed, the electrospray source of the mass spectrometer is poorly suited to effectively ionize these molecules and their fragmentation by the mass spectrometer itself was difficult. Further analysis must be performed with specific extraction methods to identify possible and useful biological activities of eumelanins produced by *Pleurotus ostreatus*.

5.6.8 Tetrahydrobiopterin

Tetrahydrobiopterin was detected in numerous samples of the wild strain. This molecule is a cofactor in the function of various enzymes, particularly aromatic amino acid hydrolases [44]. In all the samples in which tetrahydrobiopterin was found to be present, it was bound to the aromatic amino acid phenylalanine, and the presence of the phenylalanine was detected, as the mass spectrum showed a loss of 165 mass units, which corresponds to the molecular mass of phenylalanine itself. Notably, if isolated and organically synthesised, this molecule could represent a useful transporter of aromatic amino acids in subjects where these are deficient. Unfortunately, the quantities present in the fungal sample were not enough to allow its isolation and testing.

6. BIOLOGICAL ACTIVITIES OF *PLEUROTUS OSTREATUS* EXTRACT

Analyses of the biological activity of the *Pleurotus ostreatus* whole extracts were carried out with the help of the Molecular Biology Laboratory of the University of Genoa. Special thanks are due to Professor Sonia Scarfì and Dr Sara Bassi.

6.1 Materials and methods used for biological assays

6.1.1 Spectrophotometric assays

Spectrophotometric assays were made using a Beckman spectrophotometer (DU 640). The aim was to evaluate the concentration of molecules with a strong antioxidant and anti-inflammatory activity, such as phenols and flavonoids, which are known to be particularly rich in mushrooms. Associated with this research, the antioxidant potential through other tests aimed at identifying and quantifying the scavenging activity of *Pleurotus ostreatus* extracts.

6.1.2 Total phenolic content

The total phenolic content (TPC) was evaluated using the Folin-Ciocalteu assay [45]. To 100 µl of *Pleurotus ostreatus* extracts (20 mg/ml initial concentration), 800 µl of deionized water and 100 µl of Folin-Ciocalteu reagent were added. The sample was allowed to stand for 5 minutes at room temperature, after which 1 ml of Folin-Ciocalteu was added. After this, 1 ml of 10% Na_2CO_3 solution was added. After incubation for 60 minutes in the dark at room temperature, the absorbance at 550 nm was read with the spectrophotometer. The phenolic concentration was obtained by comparing the values with a calibration curve based on different gallic acid

concentrations (0.5 to 20 µg/ml) and the total phenolic content was expressed as mg gallic acid equivalent (GAE).

6.1.3 Total flavonoid content

Total flavonoid content (TFC) was measured by $AlCl_3$ colorimetric assay [46] 100 µl of each *Pleurotus ostreatus* extract (20 mg/ml initial concentration) was added to 400 µl of distilled water and 30 µl of 5% $NaNO_2$; this was allowed to stand for 5 min at room temperature. After this, 30 µl of 10% $AlCl_3$ was added to each reaction mixture and, after a further 5 minutes, 100 µl of 2 M NaOH were also added. Finally, the volume was made up to 1 ml with deionized water and the absorbance was measured at 510 nm using the spectrophotometer. Flavonoid concentration was obtained by comparing the values with a calibration curve based on different quercetin concentrations (3 to 300 µg/ml) and the total phenol content was expressed as mg quercetin equivalent (QE)

6.1.4 DPPH radical scavenging activity

The total free radical scavenging activity was assessed by the DPPH assay on each extract of *Pleurotus ostreatus.* Samples were prepared to have a final volume of 1 ml, different concentrations: 100, 250 and 500 µg/ml (initial concentration 20 mg/ml) in 250 µl of deionized water. To each sample, 500 µl of methanol and 250 µl of DPPH 2,2-diphenyl-1-picrylhydrazyl - Calbiochem®, Millipore SpA, Milan, Italy dissolved in methanol, at the final concentration of 0.2 mM, were added. A blank containing 750 µl of methanol and 250 µl of deionized water was prepared. A negative control sample containing 250 µl water, 250 µl DPPH 0.2 mM dissolved in methanol and 500 µl methanol was also prepared, while the positive control contained ascorbic acid (0.5

mg/ml, final concentration) dissolved in 250 µl water, 500 µl methanol, 250 µl DPPH 0.2 mM in methanol. In order to evaluate the possible interference of the colour of the extracts with the spectrophotometric reading, colour controls were prepared containing the 250 µl of deionized water in which the extracts were dissolved (only the highest concentration, 500 µg/ml) and 750 µl of methanol. All samples were incubated for 30 min at room temperature in the dark and then read at 517 nm using the spectrophotometer. Taking into account that in the sample used as a white, the solution with DPPH was replaced with methanol, the antioxidant activity of the samples was assessed according to their ability to quench the DPPH radical using the following equation: DPPH radical scavenging activity (%) = (A0 - A)/A0 x 100% where A represents the absorbance of the test sample and A0 represents the absorbance of the negative control. The entire procedure was performed in triplicate.

6.1.5 Cell cultures

The murine fibroblast cell line L929, the human uterine carcinoma line HeLa, the human liver carcinoma cell line HEPG2 were obtained from the American Type Culture Collection (LGC Standards srl, Milan, Italy); the human keratinocyte cell line HaCaT was obtained from Cell Lines Service (GmbH, Eppelheim, Germany). HaCaT human keratinocyte cell line was obtained from Cell Lines Service (GmbH, Eppelheim, Germany). Cells were cultured at 37°C in humidified incubator in the presence of 5% CO2 in high-glucose D-MEM (Dulbecco's modified Eagle's medium) with glutamine (Microtechsrl, Naples, Italy), to which 10% Fetal Serum was 23 added 10% Fetal Bovine Serum (Microtechsrl), as well as penicillin and streptomycin as antibiotics.

6.1.6 Statistical analysis

Statistical analyses were performed using the one-way ANOVA test followed by the Tukey test (GraphPad Software, Inc., San Diego, CA, USA). Values of $p < 0.05$ were were considered significant

6.2 Evaluation of the cytotoxicity of *Pleurotus ostreatus* extracts

The cytotoxicity of obtained *Pleurotus ostreatus* extracts was evaluated in order to determine its possible application in nutraceutical, cosmetological and potential application in pharmacological fields. Six different extracts were investigated, three from *Pleurotus ostreatus* cultivated on standard substrate and extracted in ethanol, methanol, acetonitrile, and the corresponding extracts from the fungus cultivated on lavender-enriched substrate. Evaluation of cytotoxic activity was done on murine fibroblast cell line L929, the human uterine carcinoma line HeLa, the human liver carcinoma cell line and the human keratinocyte cell line by MTT test after a 24h incubation. To guarantee the solubility of all the present molecules, the extracts were dissolved in 1% DMSO. Three different concentrations were prepared: 500 µg/mL, 100 µg/mL and 10 µg/mL for the ethanol and methanol extracts, whereas for the acetonitrile ones, which are quantitatively lower, the concentrations used were 100 µg/mL, 10 µg/mL and 1 µg/mL. Four different cell lines were tested: murine fibroblasts L929, human hepatoma cells HEPG2, human uterine carcinoma cells HeLa and human keratinocytes HaCaT. Despite the presence of some minimal differences, it was possible to verify that the cytotoxic activity of *Pleurotus ostreatus* extract was relatively low (Fig. 12,13); indeed, cell viability remained at values above 70% and

even increased in some samples. Specifically, on L929 murine fibroblasts, the only significant effect concerning the growth of the culture came from cells incubated with the highest concentration of the methanolic extract of the mushroom grown on straw, while the best result was obtained with the highest concentration of extract in acetonitrile, where a 115% increase in growth compared to the controlwas noted compared to the growth of the control. With HepG2 human hepatoma cells, no substantial variations in viability were noted between the various concentrations. Similar results regarding cytotoxicity were obtained with HeLa uterine carcinoma cells, with viability remaining constant in the presence of all the tested extracts, at all the used concentrations. It should be noted that the highest concentration of the methanolic extract, 500 µg/mL, for fungi grown on both different substrates, results in an increase in cell viability of HeLa of 132% for the extracts of *Pleurotus* grown on standard substrate and 176% for *Pleurotus* grown on substrate enriched with lavender. This increase in viability was also observed in human HaCaT keratinocyte cultures, where the highest concentrations of all three types of extract of the fungus grown on spent lavender residues resulted in a cell proliferation of 139% for the ethanolic, 166% for the methanolic and 125% for the acetonitrile extract respectively. The proliferative activity of the extracts from *Pleurotus ostreatus* grown on standard straw substrate where lower; the only significant increase was in fact obtained for the ethanolic and methanolic extracts at their highest concentration of 132% and 148% respectively.

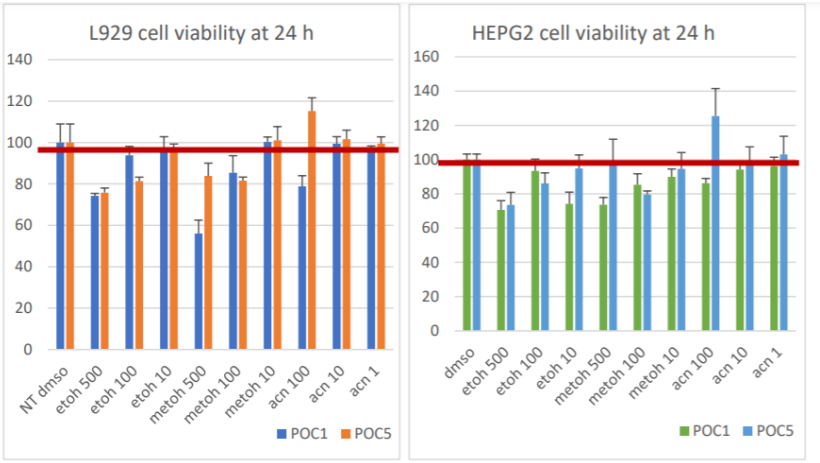


Fig. 12 MTT test of cell viability after 24 hours incubation on murine fibroblasts and human hepatoma cells with different concentrations of *Pleurotus ostreatus* whole extracts, POC1 represents the fungus grown on standard substrate and POC 5 represents the fungus grown on substrate enriched with lavender. The results are the average of two experiments in quadruplicate and are expressed as a percentage compared to the untreated control (NT DMSO)

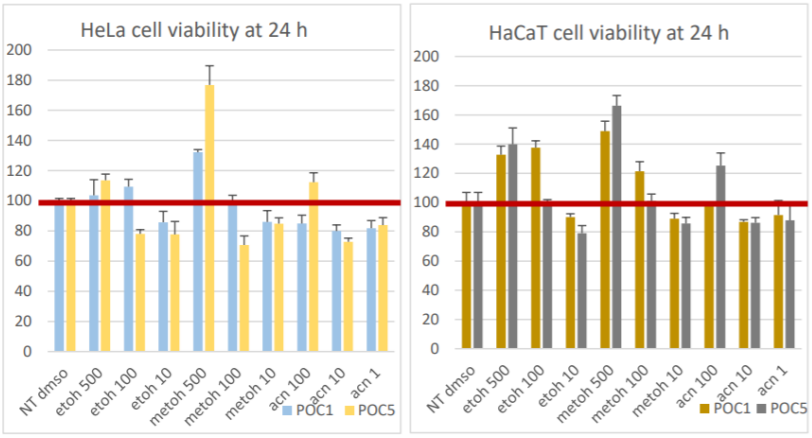


Fig. 13 MTT test of cell viability after 24 hours incubation on human uterine carcinoma cells and human keratinocytes with different concentrations of *Pleurotus ostreatus* whole extracts, POC1 represents the fungus grown on standard substrate and POC 5 represents the fungus grown on substrate enriched with lavender. Results are the mean of two experiments in quadruplicate and are expressed as percentage compared to the untreated control (NT DMSO)

6.3 Evaluation of the Total Phenolic Content and Total Flavonoid Content

The analysis of our, domestic and wild strain, *Pleurotus* samples revealed differences in the production of different primary and secondary metabolites with a clear advantage and preference over the wild strain. The growth medium proved to be a critical and essential factor for the production of numerous metabolic secondary pathways. In particular, the environment enriched with exhausted lavender extract offers a greater challenge for the survival and reproduction of a sessile fungus such as *Pleurotus ostreatus*. The way of life of fungi, that is to be heterotrophic eukaryotes organisms by absorption, or that release in the growth environment substances for the management of nutrient molecules simulation, is a determining factor for the use of these organisms as recyclers of exhausted plant waste and is also the key point that allows *Pleurotus* to be a producer of molecules with significant benefits for human health. Our approach has led to the putative identification of about 60 different molecules between primary and secondary metabolites. In particular, among the different families of compounds produced by mushrooms of great relevance to human health, the role of phenolic compounds as free radical scavengers and the role of polyphenols is fundamental, especially if we consider the polyphenols that are introduced into the body through the diet; some studies point out the inhibitory activity of polyphenols on mutagenesis and carcinogenesis in humans. The total phenolic content was evaluated using the Folin Ciocalteu assay on *Pleurotus ostreatus* extracts at a concentration of 1 mg/mL. The polyphenol content of six different *Pleurotus ostreatus* extracts obtained from different sporoma and individually extracted with the three different solvents chosen was calculated. The solvent that showed the highest extractive capacity for polyphenols is methanol, in agreement with what has been

reported for the HPLC-MS analyses. In particular, the mushroom sample grown on lavender exhausted residues gave the highest total polyphenol content of all the other samples, i.e., 31.77 µg/mL. On the other hand, polyphenols are almost completely absent in the acetonitrile extract of mushrooms grown on lavender. This is probably due to the fact that lavender is a plant rich in aromatic substances such as terpenes and terpenoids that have a protective function against fungi; these substances can therefore constitute a stressor able to induce the production of polyphenols by the fungus that uses them to protect itself from an excessive pressure effect of the environment in which it grows. However, the antioxidant activity of *Pleurotus ostreatus* is not attributable to its polyphenolic content alone; indeed, numerous other molecules with strong antioxidant activity have been identified, as polyunsaturated fatty acids, glutathione and vitamins. Nevertheless, numerous studies correlate the antioxidant activity of *Pleurotus ostreatus* with its phenolic content. The results for ethanol-extracted samples of mushrooms grown on straw and on lavender are 22.28 and 14.73 µg/mL respectively.

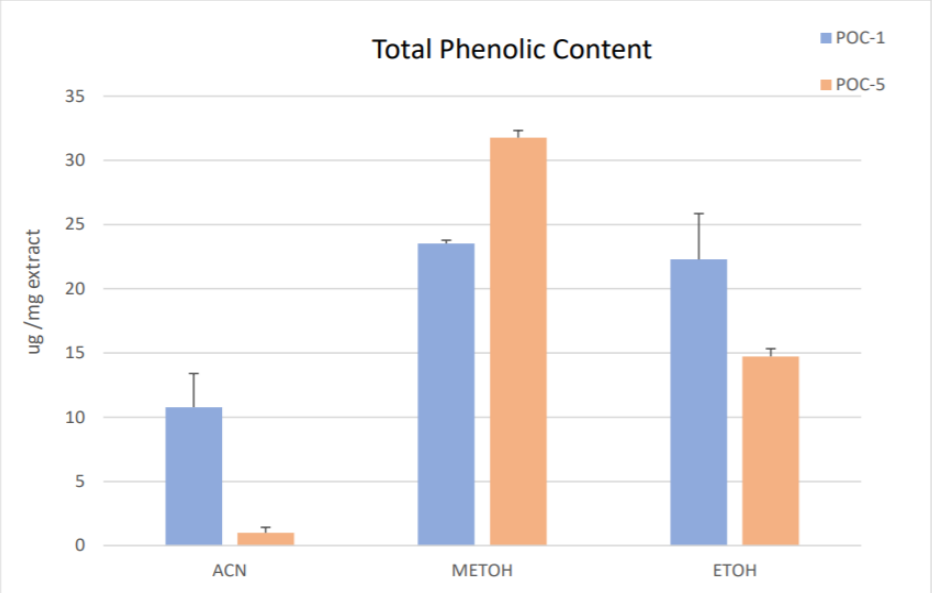


Fig. 14 Total Phenolic content determined using the Folin-Ciocalteau reagent on each of the six *Pleurotus ostreatus* extracts at a concentration of 1 mg/L. The data results are mean+/-standard deviation and are expressed as micrograms per milligram of extract.

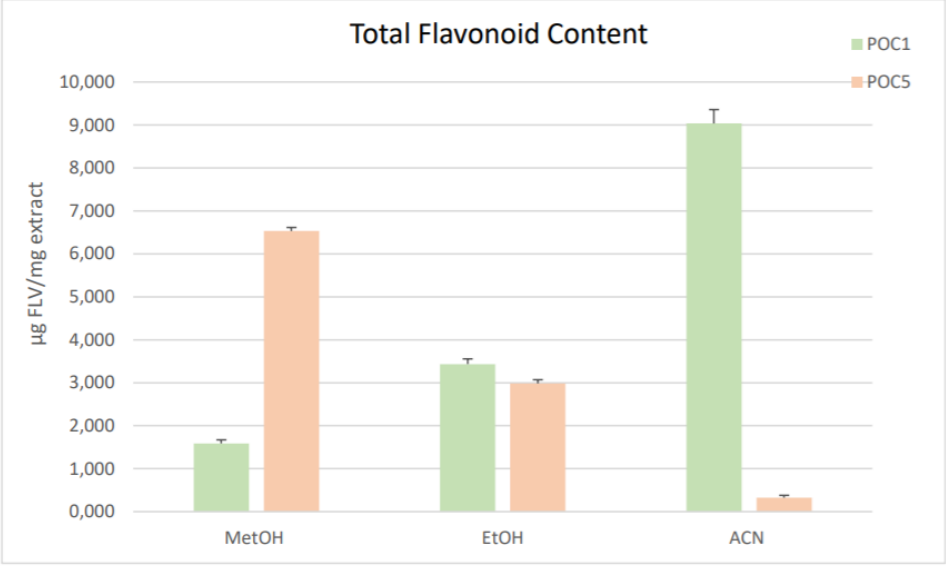


Fig. 15 Total Flavonoid Content determined using the aluminium chloride colorimetric test on each of the six *Pleurotus ostreatus* extracts at a concentration of 1 mg/mL. The data results are mean+/-standard deviation and are expressed as micrograms of flavonoids per milligram of extract.

Interestingly, there is an almost complete absence of phenols and flavonoid compounds in extracts of *Pleurotus ostreatus* enriched with lavender. This is most probably due to a use that the fungus often makes of these substances to well mitigate the effects of oxidative stress induced by the presence of terpenes and terpenoids of lavender itself. Concomitant with the decrease of phenolic compounds and flavonoids, there is an increase of molecules that are bio markers of oxidative stress such as fatty acid epoxides.

6.4 Protective action against oxidative stress-induced cytotoxicity

Cultivated mushrooms are considered an interesting source of secondary metabolites with strong antioxidant effects. This protective activity was, up to today, evaluated for several species related to *Pleurotus* ostreatus and belonging to the same genus. In this study, the cytoprotective and antioxidant effects were assessed by testing HaCaT keratinocytes to highlight the possible use of *Pleurotus ostreatus* in cosmetology. The protective action of *Pleurotus ostreatus* extracts grown on standard and non-standard substrate was evaluated in two separate experiments with two different oxidative stress agents: hydrogen peroxide and UV rays. Subsequently, rescue from induced cell death was evaluated by MTT assay [47]. The two stress agents chosen are both able to reduce cell viability in in vitro tests. In particular, hydrogen peroxide reduces cell viability by 60% compared with the control. Despite the oxidative stress damage induced on the cells, the extract of *Pleurotus ostreatus* grown on lavender resulted in an increase in cell viability of 136% compared to untreated cells and, although lower (109%), this increase in viability was also obtained for the cells treated with the highest concentration of *Pleurotus ostreatus* grown on straw.

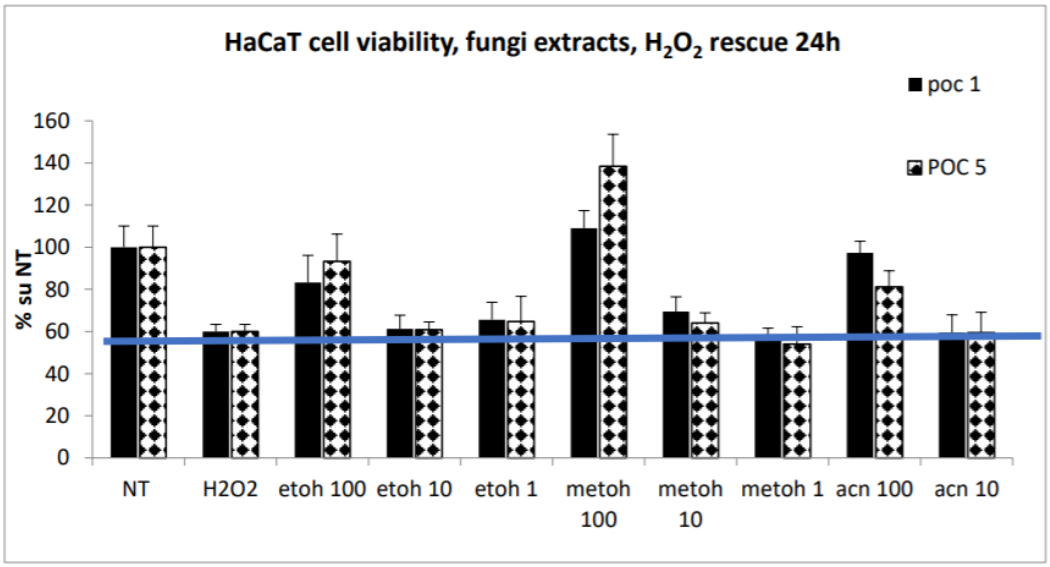


Fig. 16 Rescue of cell death in the presence of oxidative stress induced by hydrogen peroxide at a concentration of 200 micromolar on HaCat cells after 24 hours of treatment. Cells' viability was measured by MTT test. The data results are mean+/-standard deviation (n=8) and are expressed as a percentage compared to the untreated control.

The protective effect of *Pleurotus ostreatus* extracts on cells with UV-induced stress s different. There is a decrease in viability in the controls as the minutes of exposure increase, compared with the untreated control. Experiments were conducted using two different exposure times: two and five minutes. In particular, in the experiment with a shorter exposure time, a significant protective effect of the *Pleurotus ostreatus* extracts is noted, with a viability after 24 hours between 80% and 100% in the samples treated with extracts of *Pleurotus ostreatus* grown on lavender-enriched substrates which shows a remarkable protective effect against induced oxidative stress. In the experiments with a 5-minute exposure time, there is an improvement in survival exerted by all the extracts, regardless of the extraction solvent. The action of the acetonitrile extract of *Pleurotus ostreatus* cultivated on lavender-enriched substrate is particularly effective, as it improves cell survival by approximately three times respect

to the control. The particularly protective activity of this extract is most probably due to the richness of fatty acids and fatty acid epoxides it contains, which very probably act in a similar way to retinoid compounds, that are known to play an important role in the regulation, protection and cell proliferation of the human epithelium.

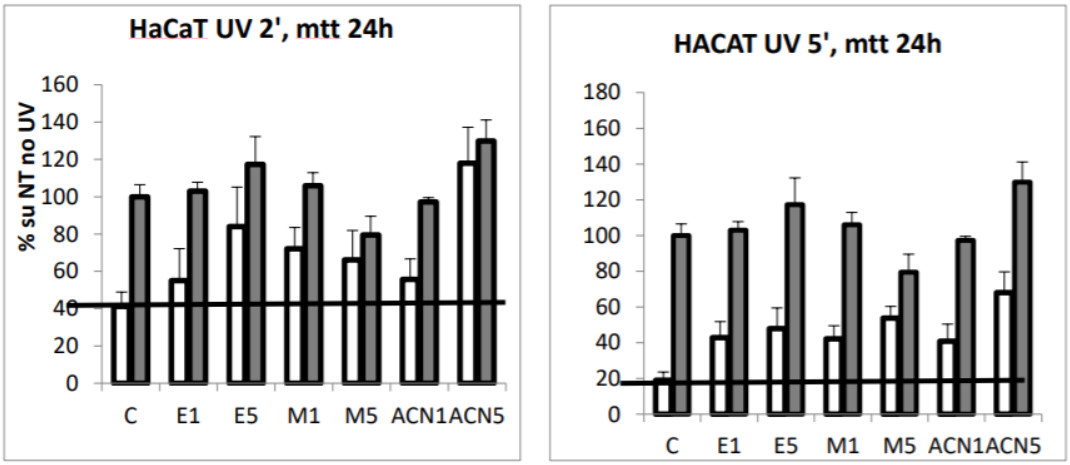


Fig. 17 Evaluation of protection from UV-induced cytotoxicity in HaCaT cells evaluated after 24 hr. The MTT test histogram shows the UV-untreated cells (dark columns) compared to the UV-treated cells (white columns). The different extracts are as follows; C is control, E1 ethanolic extract of *Pleurotus ostreatus* grown on standard substrate, E5 ethanolic extract of *Pleurotus ostreatus* grown on substrate enriched with lavender, M1 meyhanolic extract of *Pleurotus ostreatus* grown on standard substrate, M5 methanolic extract of *Pleurotus ostreatus* grown on substrate enriched with lavender, ACN1 acetonitirlic extract of *Pleurotus ostreatus* grown on standard substrate, ACN5 acetonitrilic extract of *Pleurotus ostreatus* grown on substrate enriched with lavender. The data results are mean+/- standard deviation (n=8) and are expressed as a percentage compared to the control of non-UV-exposed cells.

7 PREPARATIVE HPLC AND ISOLATION OF EXTRACT FRACTIONS

To proceed with further analysis and test of the samples, we decided to setup a separative method to be carried out in preparative HPLC. The preparative HPLC present in our laboratory works at high flows (up to 20 mL per minute) and up to 5 mL of raw extract could be injected in the column. As a raw fungal extract is extremely complex, the aim of this procedure is to have fractions of extract containing molecules belonging to different chemical families characterized by similar polarity. The columns used allow the use of high flows of eluent and are useful when large volumes of extract are involved. However, their resolving power is lower than the columns used in an analytical HPLC. It is difficult to separate the different fractions of the extract in the polar zone. The chosen method was a gradient from 0% acetonitrile to 100% acetonitrile in 45 minutes (starting from min 5), maintaining 0% of acetonitrile for the first 5 minutes and 100% of acetonitrile for further 5 minutes beyond the end of the gradient for a total of 55 minutes, with an increase of acetonitrile of 2.2% per minute. Fifteen minutes of reconditioning to bring the column at initial conditions had been added. The chosen wavelength for the UV detector were 220 nm and 254 nm. The fractions collected are as follows; the corresponding % of acetonitrile is also reported.

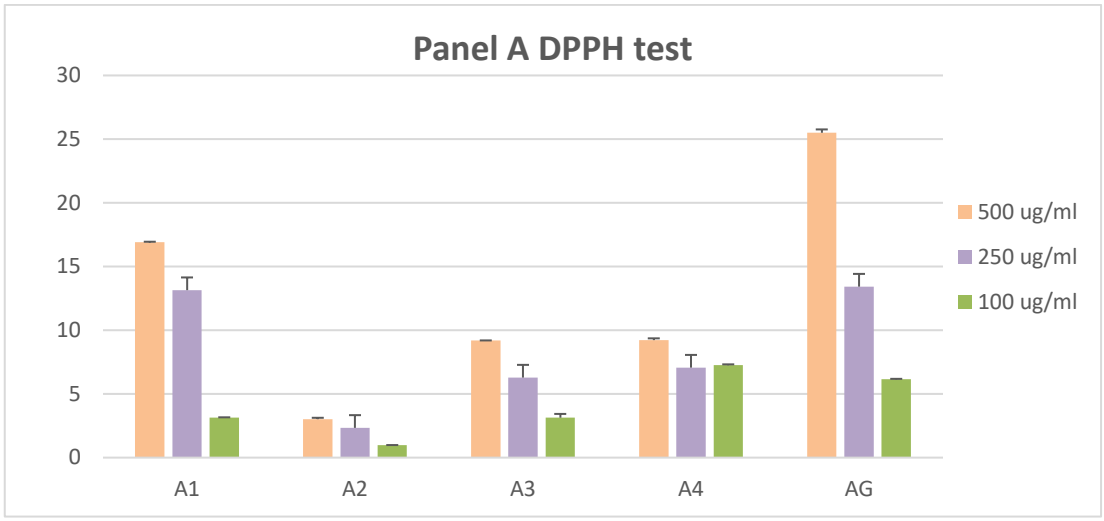
	RETENTION TIME	**% ACETONTRILE**
FRACTION 1	FROM 3 TO 10 MIN.	0 TO 22

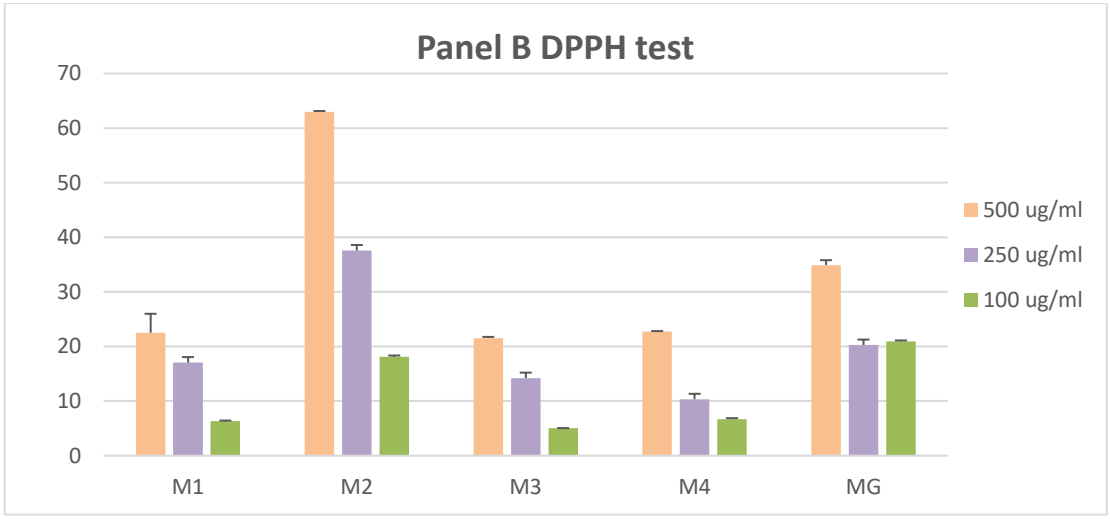
FRACTION 2	FROM 12 TO 22 MIN.	24 TO 48
FRACTION 3	FROM 25 TO 30 MIN.	55 TO 69
FRACTION 4	FROM 35 TO 40 MIN.	70 TO 100

After the collection, each fraction has been reduced in volume by a first concentration step in rotavapor and a subsequent step in SpeedVac dryer for a complete drying. The dried extracts have been weighted. The subsequent analyses regarding scavenging activity and cell viability have been conducted resuspending the extract at the concentration of 1 mg/mL in 1% DMSO followed by suitable dilution to obtain the desired concentrations.

7.1 DPPH radical scavenging activity

DPPH (2,2'-Diphenyl-1-picrylhydrazyl) [48] is a nitrogenous organic radical. The assay exploits the ability of the DPPH to be neutralized by transferring an electron or a hydrogen atom from the compound under examination in order to evaluate its antioxidant capacity. The six extracts of *Pleurotus ostreatus* were tested at different concentrations, showing overall a variable scavenging activity depending on the extraction solvent used, further demonstrating the different composition of antioxidant metabolites in several extracts. The work has been done analysing the activity of the total raw extract and comparing it to the activity of the fractionated extracts. The extracts were tested at the concentration of 500 µg/mL, 250 µg/mL, and 100 µg/mL. The results are shown in the following graphs.





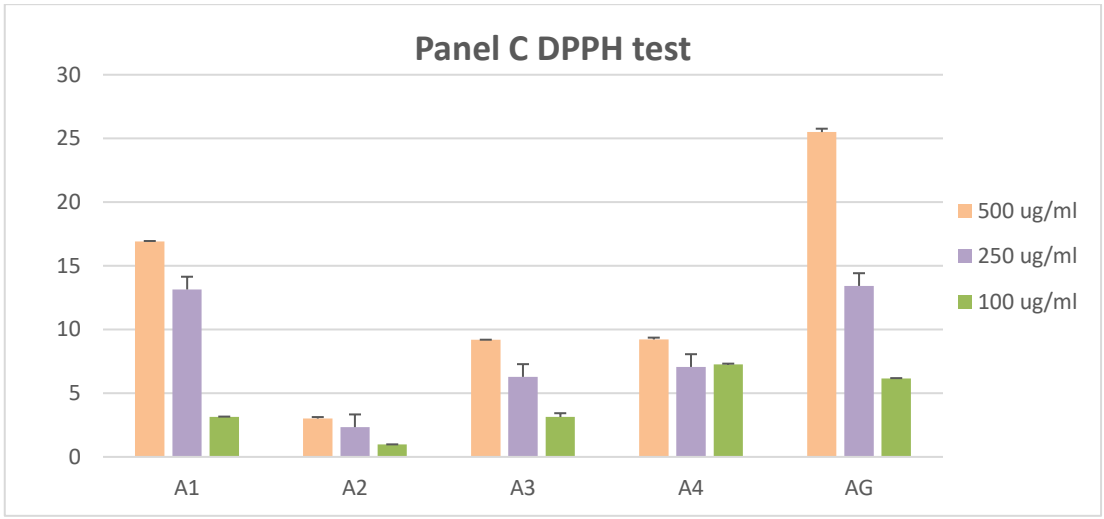


Fig. 18 panel A, B, C,: DPPH scavenging activity analyses: comparison between the different extracts and their fractions.

It can be seen that there is a variation in the results obtained from the raw extract with respect to the different fractions of each extract. In particular, it can be noted that the different fractions often possess considerably different activities from each other, in some cases promoting the scavenging of free radicals, in other cases showing even lower activity compared to the total extract. The best results were obtained with the ethanol and methanol extracts in fractions one and two. Indeed, the polyphenolic compounds that have been detected in HPLC-MS analyses are concentrated in these fractions. The free radical scavenging activity of fungal extracts must therefore be attributed more to these classes of compounds. As regards to the acetonitrile extracts, it has to be noted that the total raw extract has a scavenging activity higher than that of its fractions although, in absolute, it is lower than that of the ethanol and methanol extracts. This is due to the lack of polar compounds present in the extract, which is therefore lacking in polyphenols and other families of compounds with antioxidant activity, while it is rich in free fatty acids and epoxides of fatty acids.

7.2 Cell Viability tests on fractionated extracts

In cell viability tests conducted, human keratinocytes proved to be the cell line giving the most interesting results for testing fungal extracts. This is probably due to the high metabolism and fast proliferation, typical characteristics of these cells. Extracts were tested at different concentrations, 100 µg/mL, 10 µg/mL and 1 µg/mL. The data obtained in relation to the untreated cells are very encouraging but different according to the extract.

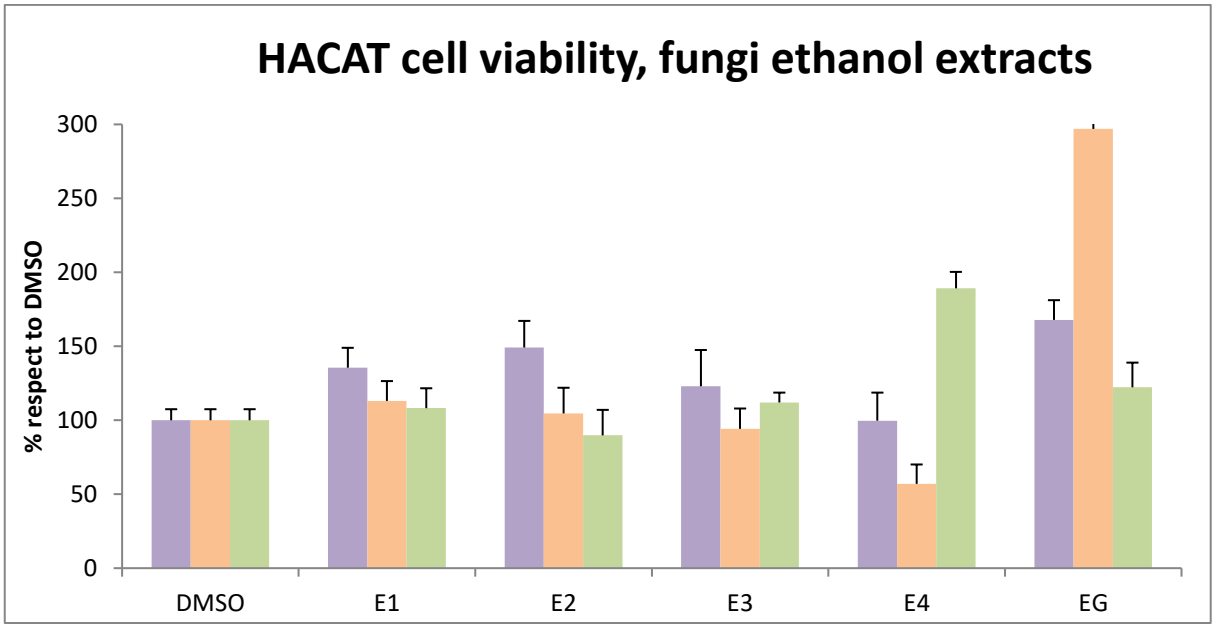


Fig. 19 Cell viability tests after 24 h obtained with total raw methanolic extract and the 4 fractions in comparison to non-treated cells. The extracts had been used at different concentrations (purple 100 µg/mL, orange 10 µg/mL and green 1 µg/mL). E refers to ethanolic extract, 1,2,3,4 are the fractions of the extract in reference to table presented at pag.70,71 and EG is the raw extract for comparison of activity.

In the ethanol extract it can be noted that the crude extract has a cell growth promoting activity higher than any of its fractions. This is very interesting since it would indicate that different molecules of the ethanol extract produced by the fungus could contribute in synergy to increase cell proliferation. No substantial differences in cell viability are revealed, except a lower viability after 24 hours, of the cells exposed to fractions three and four: likely, this is due to the fact that fatty acid epoxides (which, as reported in the literature, are molecules that show a marked cytotoxic activity) are present in these fractions.

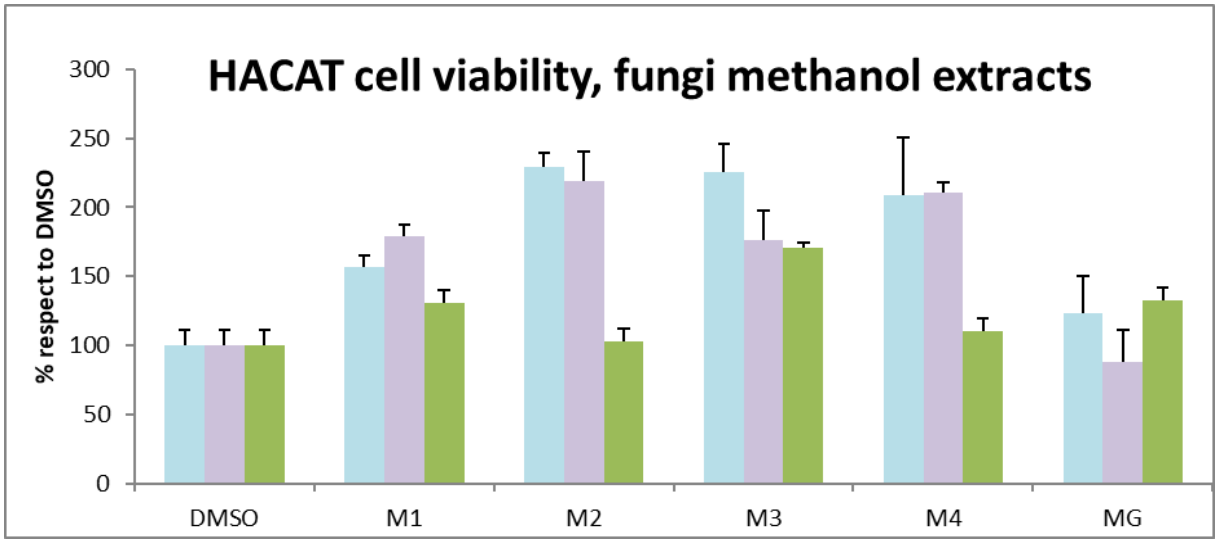


Fig. 20 Cell viability tests after 24 h obtained with total raw methanolic extract and the 4 fractions in comparison to non-treated cells. The extracts had been used at different concentrations (blue 100 µg/mL, lilac 10 µg/mL and green 1 µg/mL). M refers to methanolic extract, 1,2,3,4 are the fractions of the extract in reference to table presented at pag.70,71 and MG is the raw extract for comparison of activity.

Cell viability tests conducted with fractionated methanol extracts gave the most interesting results. This agrees with the data observed in previous analyses: the methanolic extracts are the richest in quantity and quality of the substances present. Moreover, they are missing several molecules with probable cytotoxic activity. The fractions obtained separating the methanol extracts demonstrate an excellent ability to increase cell growth up to 250% - 270% and there are no major differences between the fractions two, three and four. It should be noted that the raw methanol extract does not present the same beneficial activity in the proliferation of keratinocytes. This is probably due to an anti-synergistic action of the different compounds contained in it. Interesting, from a biological point of view, is the richness of Amadori compounds present in this type of extract. In the mushrooms Amadori compounds of isoleucine,

leucine, alanine and phenylalanine are naturally produced, as it is the method of recovery of essential amino acids through the degradation of old proteins, and their presence could have a great therapeutic significance. As reported in literature, Amadori compounds shows a high antioxidant activity and their natural presence in *Pleurotus ostreatus* extracts should be considered as a further added value, just for their efficacy as molecular complexes with beneficial activities for human cells [49]

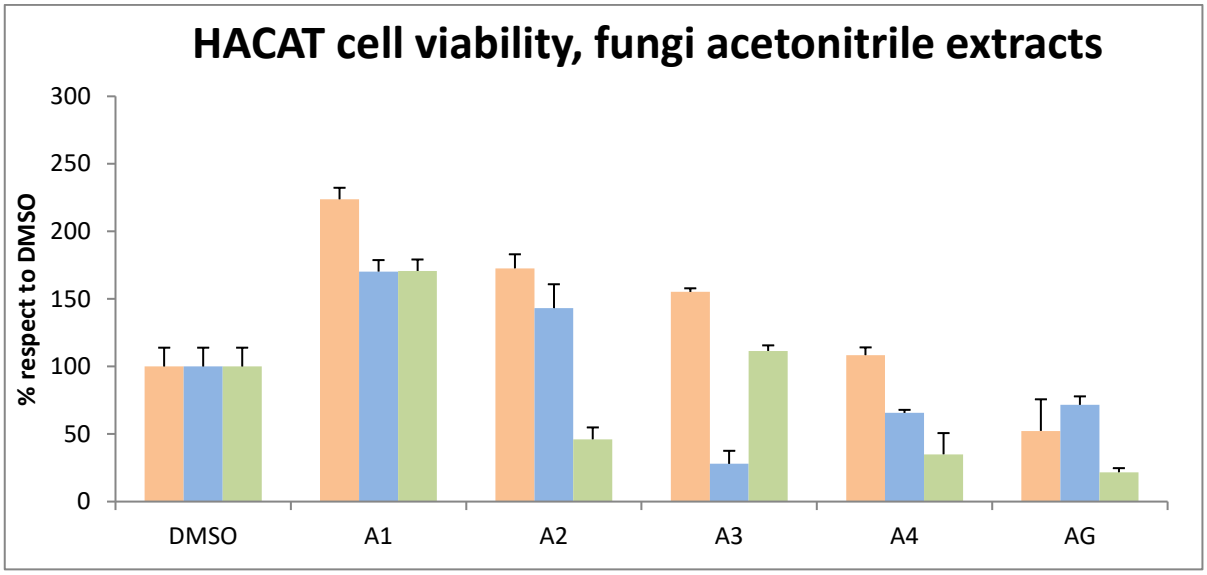


Fig. 21 Cell viability tests after 24 h obtained with total raw acetonitrile extract and the 4 fractions in comparison to non-treated cells. The extracts had been used at different concentrations (orange 100 µg/mL, navy 10 µg/mL and green 1 µg/mL). A refers to acetonitrilic extract, 1,2,3,4 are the fractions of the extract in reference to table presented at pag.70,71 and AG is the raw extract for comparison of activity.

In the fractions obtained from acetonitrile extracts there is a negative effect on cell growth; in particular the fractions two, three and four, the richest in fatty acid epoxides, showed a fair cytotoxic activity with results in agreement with the data obtained performing antioxidant activity tests and with HPLC-MS analyses. However, it is interesting to note that fraction one, in which the fatty acids are not present, has a positive effect on keratinocytes growth, with very appreciable results in all three concentrations tested.

8 CONCLUSIONS

This work has highlighted how the growth substrate is of fundamental importance as the interface of the fungus with its environment and how it directly influences the composition of the secondary and primary metabolism of the fungus itself. It has been possible to provide a more complete view of the primary and secondary metabolomics of *Pleurotus ostreatus* using a method of extraction and analysis in HPLC-MS and tandem mass that was not limited to the analysis of a specific class of chemical compounds. *Pleurotus ostreatus* is confirmed to be an interesting fungal species from the point of view of the production of molecules with powerful antioxidant, anti-inflammatory, protective and cell proliferative effects. The extracts were carefully analyzed by means of tests that highlighted their beneficial action. In particular, tolerance in terms of cytotoxicity has been validated on different cell lines, demonstrating that *Pleurotus ostreatus* and its extracts have very low toxicity. Interesting results were obtained on human keratinocytes. The keratinocytes are the most abundant cell type in the epidermis, present in all its layers, and represent the cellular life stages of the keratinocyte itself. Since they are a major component of our epidermis, the keratinocyte is also the human body's first interface with the external environment and is therefore particularly susceptible to damage from UV radiation from the sun and from damage caused by atmospheric agents and pollution. The effects of the extracts tested on human keratinocytes showed how fungal metabolites are able to act at different levels of the damage process, even preventing it or acting as a repair agent. All the data obtained show how *Pleurotus ostreatus* extracts, with particular reference to those obtained using methanol and acetonitrile from fungi cultivated on lavender-enriched substrates, have significantly greater effects than those of other

extracts, indicating that *Pleurotus ostreatus* grown on the lavander may be a new source of promising molecules to be included in various types of cosmetic and nutraceutical formulations. All the analyses carried out lay the foundations for continuing the study on specific compounds, and new analytical and preparative methods will be developed to enable the separation of individual molecules. New tests will then be carried out on these cell lines under the same conditions to identify which molecule or molecules present in the extracts are most effective and whether there are synergistic actions between different compounds. The unambiguously demonstrated low cellular toxicity opens the way to the formulation of antioxidant nutraceutical preparations for anti-ageing purposes and cosmetic lotions that can soothe and repair damaged epidermis.

BIBLIOGRAPHY

[1] Vasileva I., Denkova R., Chochkov R., Teneva D., Denkova Z., Dessev T., Denev P., Slavov A. (2018) Effect of lavender (Lavandula angustifolia) and melissa (Melissa officinalis) waste on quality and shelf life of bread. Food Chem. Jul 1;253:13-21. doi: 10.1016/j.foodchem.2018.01.131.

[2] Oludemi T., Martins A., Barreiro M.F. &. Ferreira I., (2016). Anti-Inflammatory Potential of Mushroom Extracts and Isolated Metabolites. - Trends in Food Science and Technology 50: 193–210.

[3] Sułkowska-Ziaja K., Muszyńska B., Końska G. (2005) Biologically active compounds of fungal origin displaying antitumor activity. Acta Pol Pharm. Mar-Apr;62(2):153-9.

[4] Sánchez, C., (2010). Cultivation of Pleurotus ostreatus and other edible mushrooms. Appl Microbiol Biotechnol 85, 1321–1337 https://doi.org/10.1007/s00253-009-2343-7

[5] Hawksworth D.L., &Lücking R. (2017) Fungal Diversity Revisited: 2.2 to 3.8 Million Species. Microbiol Spectrum, 1–17 doi: 10.1128/microbiolspec.FUNK-0052-2016

[6] Keeling, P.J.; Doolittle W.F., (1996(. "Alpha-Tubulin from Early-Diverging Eukaryotic Lineages and the Evolution of the Tubulin Family." Molecular Biology and Evolution 13 (10): 1297–1305.

[7] Stamets P, Zwickey H., (2014). Medicinal Mushrooms: Ancient Remedies Meet Modern Science. Integr Med (Encinitas) (1):46-7.

[8] Beulah G., Hepzibah A., Margret A., and Jeyakumar N., (2013). MarvelousMedicinal Mushrooms." nternational Journal of Pharmacy and Biological

[9] Van Dyk M.S., Kock J.L.F. & Botha, A. (1994). Hydroxy long-chain fatty acids in fungi. World J Microbiol Biotechnol 10, 495–504 https://doi.org/10.1007/BF00367653

[10] Cardwell G., Bornman J.F., James A.P., Black L.J. (2018). Review of Mushrooms as a Potential Source of Dietary Vitamin D. Nutrients.;10(10):1498. doi:10.3390/nu10101498

[11] Sun J. (2018). Dietary vitamin D, vitamin D receptor, and microbiome. Curr Opin Clin Nutr Metab Care. Nov;21(6):471-474. doi: 10.1097/MCO.0000000000000516.

[12] Hallen-Adams H.E., Suhr M.J. (2017). Fungi in the healthy human gastrointestinal tract. Virulence. Apr 3;8(3):352-358. doi: 10.1080/21505594.2016.1247140

[13] Lull C., Wichers H.J., Savelkoul H.F., (2005) Antiinflammatory and immunomodulating properties of fungal metabolites. Mediators Inflamm :63-80. doi: 10.1155/MI.2005.63.

[14] Chang S.T., Wasser S.P., (2018) Current and Future Research Trends in Agricultural and Biomedical Applications of Medicinal Mushrooms and Mushroom Products (Review). Int J Med Mushrooms.;20(12):1121-1133. doi: 10.1615/IntJMedMushrooms.2018029378.

[15] Volman J.J., Helsper J.P., Wei S., Baars J.J., van Griensven L.J., Sonnenberg A.S., Mensink R.P., Plat J., (2010) Effects of mushroom-derived beta-glucan-rich polysaccharide extracts on nitric oxide production by bone marrow-derived macrophages and nuclear factor-kappaB transactivation in Caco-2 reporter cells: can effects be explained by structure? Mol Nutr Food Res. Feb;54(2):268-76. doi: 10.1002/mnfr.200900009.

[16] Wasser S.P., (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. Appl Microbiol Biotechnol. Nov;60(3):258-74. doi: 10.1007/s00253-002-1076-7.

[17] Bakir T., Karadeniz M., Unal S., (2018). Investigation of antioxidant activities of Pleurotus ostreatus stored at different temperatures. Food Sci Nutr. Apr 16;6(4):1040-1044. doi: 10.1002/fsn3.644. PMID: 29983968.

[18] Guo Y.J., Deng G.F., Xu X.R., Wu S., Li S., Xia E.Q., Li F., Chen F., Ling W.H., Li H.B., (2012) Antioxidant capacities, phenolic compounds and polysaccharide contents of 49 edible macro-fungi. Food Funct. Nov;3(11):1195-205. doi: 10.1039/c2fo30110e.

[19] Muszyńska B., Grzywacz-Kisielewska A., Kała K., Gdula-Argasińska J., (2017). Anti-inflammatory properties of edible mushrooms: A review. Food Chem. Mar 15;243:373-381. doi: 10.1016/j.foodchem.2017.09.149.

[20] Resende D.I.S.P., Almeida M.C., Maciel B., Carmo H., Sousa Lobo J., Dal Pozzo C., Cravo S.M., Rosa G.P., Kane-Pagès A., do Carmo Barreto M., Almeida I.F., de Sousa M.E., Pinto M.M.M., (2020) Efficacy, Stability, and Safety Evaluation of New Polyphenolic Xanthones Towards Identification of Bioactive Compounds to Fight Skin Photoaging. Molecules. Jun 16;25(12):2782. doi: 10.3390/molecules25122782.

[21] Keswani C., Singh H.B., Hermosa R., García-Estrada C., Caradus J., He Y.W., Mezaache-Aichour S., Glare T.R., Borriss R., Vinale F., Sansinenea E., (2019). Antimicrobial secondary metabolites from agriculturally important fungi as next biocontrol agents. Appl Microbiol BiotechnolDec;103(23-24):9287-9303. doi: 10.1007/s00253-019-10209-2.

[22] Damonte G., Salis A., Rossi L., Magnani M., Benatti U., (2007) High throughput HPLC-ESI-MS method for the quantitation of dexamethasone in blood plasma. https://doi.org/10.1016/j.jpba.2006.06.042.

[23] Frisvad C., (1987). High-performance liquid chromatographic determination of profiles of mycotoxines and other secondary metabolites. https://doi.org/10.1016/S0021-9673(01)94277-3.

[24] Craig M., Whitehouse R.N., Dreyer M.Y., Fenn J.B., (1994). Electrospray interface for liquid chromatographs and mass spectrometers. https://doi.org/10.1021/ac00280a023

[25] Carvalho Gomes Correa R., Brugnari T., Bracht A., Rosane M. P., Isabel C.F.R. Ferreira (2016). Biotechnological, nutritional and therapeutic uses of Pleurotus spp. (Oyster mushroom) related with its chemical composition: A review on the past decade findings doi: http://dx.doi.org/10.1016/j.tifs.2016.01.0120924-2244/

[26] Scalbert a., Johnson I.T., & Saltmarsh M., (2005). Polyphenols: antioxidants and beyond The American Journal of Clinical Nutrition, Volume 81, Issue 1, Pages 215S–217S, doi: https://doi.org/10.1093/ajcn/81.1.215S

[27] Gasecka M., Mleczek M., ·Siwulski M., Niedzielski P., (2016). Phenolic composition and antioxidant properties of Pleurotus ostreatus and Pleurotus eryngii enriched with selenium and zinc 242:723–732 DOI: 10.1007/s00217-015-2580-1

[28] Bano, Z. & Rajarathnam, S. (1986). Vitamin values of Pleurotus mushrooms Plant Food Hum Nutr 36: 11. https://doi.org/10.1007/BF01091748

[29] Rajarathnam S.Z. & Steinkraus K.H. (2009). Pleurotus mushrooms. Part II. Chemical composition, nutritional value, post-harvest physiology, preservation, and role as human food Pages 87-158 2009 https://doi.org/10.1080/10408398809527480

[30] Ginterová A., Lazarová A. (1987). Amino acid composition of wood-rotting fungi (Pleurotus) and total amino acid balance of the cultivating system. Food Chemistry Volume 23, Issue 1, 1987, Pages 35-41 https://doi.org/10.1016/0308-8146(87)90025-2

[31] Tolera K.D., Abera S. (2017) Nutritional quality of Oyster Mushroom (Pleurotus Ostreatus) as affected by osmotic pretreatments and drying methods. ;5(5):989–996. doi:10.1002/fsn3.484

[32] Shahdat H., A., Amin N., Basunia S.M., Rahman M. (2007). Essential fatty acid contents of Pleurotus ostreatus, Ganoderma lucidum and Agaricus bisporus. Bangladesh Journal of Mushroom. 2. 1-7

[33] Jose N., Ajith T.A., Janardhanan K.K. (2002). Antioxidant, antiinflammatory, and antitumor activities of culinary-medicinal mushroom Pleurotus pufmonanus (Fr.) Quel. (Agaricomycetideae). Int J Med Mushrooms 4:329–335. doi: 10.1615/IntJMedMushr.v4.i4.60

[34] Willcox JK., Ash S.L., Catignani G.L. (2004). Antioxidants and prevention of chronic disease. Rev Food Sci Nutr 44:275–295 doi: 10.1080/10408690490468489

[35] Reis F.S., Martins A., Barros L., Ferreira I., (2012). Antioxidant properties and phenolic profile of the most widely appreciated cultivated mushrooms: A comparative study between in vivo and in vitro samples, Food and Chemical Toxicology, Volume 50, Issue 5,Pages 1201-1207, ISSN 0278-6915Nov 9. pmid: 31707442.

[36] Hlavica P. (2006). Functional interaction of nitrogenous organic bases with cytochrome P450: a critical assessment and update of substrate features and predicted key active-site elements steering the access, binding, and orientation of amines. Biochim Biophys Acta. Apr;1764(4):645-70. doi: 10.1016/j.bbapap.2006.01.013.

[37] Ortiz A, Sansinenea E. (2007). Cyclic Dipeptides: Secondary Metabolites Isolated from Different Microorganisms with Diverse Biological Activities. Curr Med Chem.;24(25):2773-2780. doi: 10.2174/0929867324666170623092818.

[38] Pednault K., Angers P., Tyler J. A., Gosselin A., Tweddell R. J. (2007) Fatty acid profiles of polar and non-polar lipids of Pleurotus ostreatus and P. cornucopiae var. 'citrino-pileatus' grown at different temperatures: doi:10.1016/j.mycres.2007.06.014

[39] Kennedy M.J., Reader, Sarah L, Davies, R. J. (1993). Fatty acid production characteristics of fungi with particular emphasis on gamma linolenic acid production doi: 10.1002/bit.260420511 Biotechnology and bioengineering.,

[40] Carvalho Gomes Correa R., Brugnari T., Bracht A., Rosane M. P., Isabel C.F.R. Ferreira (2016). Biotechnological, nutritional and therapeutic uses of Pleurotus spp.

(Oyster mushroom) related with its chemical composition: A review on the past decade findings doi: http://dx.doi.org/10.1016/j.tifs.2016.01.0120924-2244/

[41] Node, K., Huo, Y., Ruan, X., Yang, B., Spiecker, M., Ley, Liao, J. K. (1999). Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. Science, 285(5431), 1276-1279. doi:10.1126/science.285.5431.1276y

[42] Helliwell K.E. (2017) The roles of B vitamins in phytoplankton nutrition: new perspectives and prospects. New Phytol. 2017 Oct;216(1):62-68. doi: 10.1111/nph.14669.

[43] Langfelder K., Streibel M., Jahn B., Haase G, Brakhage A.A. (2003) Biosynthesis of fungal melanins and their importance for human pathogenic fungi. Fungal Genet Biol. Mar;38(2):143-58. doi: 10.1016/s1087-1845(02)00526-1.

[44] Flydal M.I., Martinez A. (2013) Phenylalanine hydroxylase: function, structure, and regulation. IUBMB Life. 2013 Apr;65(4):341-9. doi: 10.1002/iub.1150.

[45] Agbor G. & Vinson J. & Donnelly P. (2014). Folin-Ciocalteau Reagent for Polyphenolic Assay. International Journal of Food Science, Nutrition and Dietetics. 3:801. 1

[46] John, B. & C T, Sulaiman & George, Satheesh & Reddy, (2014). Total phenolics and flavonoids in selected medicinal plants from Kerala. International Journal of Pharmacy and Pharmaceutical Sciences. 6. 406-408.

0.19070/2326-3350-1400028.

[47] van Meerloo J, Kaspers GJ, Cloos J. Cell sensitivity assays: the MTT assay. Methods Mol Biol. 2011; 731:237-45. doi: 10.1007/978-1-61779-080-5_20

[48] Bondet, V., Brand-Williams, W. and Berset, C. (1997) Kinetics and Mechanisms of Antioxidant Activity Using the DPPH Free Radical Method. LWT-Food Science and Technology, 30, 609-615. http://dx.doi.org/10.1006/fstl.1997.0240

[49] Yoshida N., TakatsukaK., Katsuragi T. & Tani Y.(2005) Occurrence of Fructosyl-Amino Acid Oxidase-Reactive Compounds in Fungal Cells, Bioscience, Biotechnology, and Biochemistry, 69:1, 258-260, DOI: 10.1271/bbb.69.258

ACKNOWLEDGEMENTS

My heartfelt thanks:

to Professor Gianluca Damonte and Dr. Annalisa Salis for all the teachings, support and help.

To Professor Mirca Zotti, Dr. Simone DiPiazza and Dr. Grazia Cecchi for the support and collaboration

To Professor Sonia Scarfì for the invaluable help