Determination of the different phenolic and volatile compounds involved in the mycorrhizal process in vitro

Rayda Mezlini

Dissertation Presented to the School of Agriculture of the Polytechnic Institute of Bragança to obtain the Master Degree in Biotechnological Engineeringy, in the scope of the double degree with free university of Tunisia

Supervised by

Anabela Martins

Maria João Sousa

This Dissertation takes into account the changes and suggestions made by the Jury

BRAGANÇA

JULY 2021

Acknowledgments

I would like to show my sincere appreciation to those who have contributed to this dissertation and supported me in one way or the other during this amazing journey for without the generous support of them, this work would not have been possible.

First of all, I would like to express my pride to be supervised by widely known and highly experienced scientific researchers.

Professora Anabela Martins and **Professora Maria João Sousa,** I am very thankful to have such a wonderful, professional and perfectionist persons as my thesis supervisors. I am deeply thankful for your continuous support and patience in this learning path, for your persistent help, and for the constant motivation to do more and better. You offered continuous advices and encouragement and I benefitted from your knowledge and scientific experience. I appreciate your effort and I want to thank you for your wise guidance, kindness and continuous availability.

Doctor Filipa Reis my ultimate support and mentor. I am so grateful for everything that I have learned from you during our lab work time, your irreplaceable guidance and great help in laboratory procedures; you are a kind person with a warm loving heart. Your enthusiasm and energy that I admire made the lab a wonderful place to work in and created the best environment ever to make up for the distance. Your positive outlook and your ability to smile despite the situation is your special super power. You are a wonderful and beautiful person both inside and out; I would like to express my gratitude for all your support and encouragement. Thank you for always finding the time to guide me through all the difficult moments of research and while writing this thesis, your support and words of encouragement gave me the power to commit myself to this work.

Thank you for always being so friendly and supportive with your smile and generous words of encouragement. You were there by my side on every step of my path and for everything you've done for me I'm so glad to consider you as a **FRIEND**.

I also want to thank all researchers of the Mountain Research Centre (CIMO) for their support and generosity. Thank you all for always being so helpful and friendly, as well as for all your efforts and support during the realization of this work.

I am deeply grateful to all members of the jury who kindly honoured me by their presence to participate in the defence of this thesis.

My big appreciation to my special and wonderful friends, who helped me in every possible way to achieve my dreams and successfully complete this research work. I will never forget all their support and effort. You're the best.

Keeping the best for last, I would like to thank my parents **Ridha Mezlini, Rim Louizi** and my sisters **Rabeb** and **Roua** whose help made this journey possible from the beginning they believe in me and supported my studies abroad both morally and financially. They have been my inspiration and my unfailing source of passion and energy. Thank you for your irreplaceable support, for all your love, patience and kindness, for always encouraging me to pursuit my education, for believing in me and for everything that you have done to make me the person I am now. Without you none of this would have been possible. I love you!!

INDEX

List of figures

List of tables

List of abbreviations

AAPH	Dihydrochloride
AOAC	Association of analytical communities
ANOVA	Analysis of variance
DAD	Diode Array Detector
DMEM	Dulbecco'sModifiedEagle Medium
DMM	Dimethyl malonate
DMSO	Dimethyl sulfoxide
DO	Optical Density
dw	Dry Weight
ECM	Ectomycorrhizas
EC	European Commission
ESI	Electrospray ionization
FAME	Fatty acid methyl ester
FID	Flame ionozation detectors
HPLC	High Performance Liquid Chromatography
HeLa	Cervical Carcinoma
HepG2	Hepatocellular Carcinoma
IS	Internal standard
LC	Liquid chromatography
L-N	Licken-Nickerson
MBC	Minimal Bactericidal Concentration
MDA	Malondialdehyde
MUFA	Monounsaturated fatty acids;
MIC	Minimal Inhibitory Concentration
nd	Not detected
NM	Non mycorrhizal
PBS	Phosphate Buffered Saline
PDB	Protein Data Bank
PLP2	Porcine Liver Primary Culture
PE	Percentage of the erythrocyte
PTGS	Prostaglandin endoperoxide synthase
PUFA	Polyunsaturated fatty acids

RAW 264.7	Murine Macrophage Cell Line
RNS	Reactive Sulphate species
SRB	Sulforhodamine B
SFA	Saturated fatty acids
TCA	Tricarboxylic acid cycle
TBA	Thiobarbituric acid
TBARS	Thiobarbituric Acid Reactive Species
TSB	Tryptic Soy Broth
Tr	Traces
UFC	Unit Forming colony
UFLC	Ultra-Fast Liquid Chromatography
UPLC	Ultra Performance Liquid Chromatography

ABSTRACT

The roots of most terrestrial plants are colonized by mycorrhizal fungi. Mycorrhizae are symbiotic associations established between certain soil fungi and most vascular plants worldwide. Mycorrhizal symbioses improve the resilience of plant communities against environmental stresses, including nutrient deficiency, in particular increase the roots' ability to better capture nutrients and avoid soil disturbance. The plant produces organic molecules such as sugars by photosynthesis and supplies them to the fungus, and the fungus supplies to the plant water and mineral nutrients, such as phosphorus, taken from the soil. Through mycorrhization, the plant obtains phosphate and other minerals, such as zinc and copper, from the soil.

Thus, during this process there is the release of compounds from the secondary metabolism, some of them characterized by their bioactive potential. Phenolic compounds and volatile compounds (secondary metabolites) are some of the molecules released during the oxidative stress process.

To better understand the establishment of the mycorrhizal symbiosis and to promote the production of compounds of interest, namely secondary metabolites, the plant *Castanea sativa* Mill and the mycorrhizal fungi *Paxillus involutus* (Batsch) Fr. were placed in co-culture for different periods (24h and 48h). The evaluation of phenolic and volatile compounds in both species was evaluated before and after the co-culture, the aim of the present work was to (i) induce the mycorrhizal symbiosis of chestnut tree with compatible fungi *in vitro,* and (ii) search for phenolic and volatile compounds involved in the mycorrhizal process *in vitro*, (iii) analyzing the influence of the contact with the host roots on the profile of these compounds. After the co-culture period, the mushroom and the roots of *C. sativa* were analysed for the presence of phenolic and volatile compounds. The phenolic compounds were assessed by liquid chromatography equipped with a diode-array detector and coupled to a mass detector (LC-DAD-ESI/MSn). The volatile compounds, after extraction by hydrodistillation, will be determined by GC-MS (Gas Chromatography - mass spectrometry).

The results obtained, showed the same phenolic compounds in the control roots and in the roots co-cultured with the mycorrhizal fungus (24h and 48h). Regarding the fungus (*P. involutus*), the identification was very difficult, but one fragments of compounds were present, namely acids (phenylethanol).

Wild mushrooms have been pointed as valuable health foods, rich in protein, vitamins and minerals; a source of bioactive compounds, such as unsaturated fatty acids or mono/oligosaccharides; and as having bioactive properties, such as antioxidant, anti-inflammatory, or antimicrobial activities.

During the collection of mushrooms for the present work, other than isolation *in vitro* : the analysis of wild mushrooms (*Lactarius deliciosus , Boletus pinicola 2, Boletus pinicola 3, Tricholoma terreum* and *Tricholoma equestre*) in order to evaluate the antioxidant potential (free radical scavenging effect, reducing power and inhibition of lipid peroxidation), antimicrobial (antibacterial using Gram-positive and Gram-negative strains and antifungal activity) and the absence of toxicity (porcine liver primary cells) of the ethanol/methanol extracts, anti-inflammatory activity, and to contribute with updated information on natural sources of bioactive compounds, thorough chemical characterization and possible applications.

The results obtained demonstrated that the extract of all wild mushrooms studied presented antioxidant activity, antimicrobial, cytotoxity activities and contained different classes of various bioactive molecules, besides carbohydrates lipids, proteins, and minerals. For the results of the phenolic compounds, the five mushroom samples presented 2 phenolic compounds protocatechuic acid and *p*-hydroxybenzoic acid.

Keywords

Mycorrhizal symbiosis, *Castanea sativa* , *Paxillus involutus* , edible mushrooms; *Lactarius deliciosus, Boletus pinicola 2, Boletus pinicola 3, Tricholoma terreum*; *Tricholoma equestre*, secondary metabolites, phenolic compounds, volatile compounds, bioactive compounds, antioxidant, anti-inflammatory, antimicrobial activities.

RESUMO

As raízes da maioria das plantas terrestres são colonizadas por fungos micorrízicos. As micorrizas são associações simbióticas estabelecidas entre certos fungos do solo e a maioria das plantas vasculares em todo o mundo. As simbioses micorrízicas melhoram a resiliência das comunidades de plantas contra os stresses do meio ambiente, incluindo a deficiência de nutrientes, em particular aumentam a capacidade das raízes de capar melhor os nutrientes e evitar perturbações do solo. A planta produz moléculas orgânicas como açúcares por fotossíntese e fornece-as ao fungo, e o fungo fornece à planta água e nutrientes minerais, como fósforo, retirados do solo. Por meio da micorrização, a planta obtém fosfato e outros minerais, como zinco e cobre, do solo.

Assim, durante esse processo ocorre a libertação de compostos do metabolismo secundário, caracterizado pelo seu potencial bioativo. Compostos fenólicos e compostos voláteis (metabolitos secundários) são algumas das moléculas libertadas durante o processo de stresse oxidativo.

Para melhor compreender o estabelecimento da simbiose micorrízica e promover a produção de compostos de interesse, nomeadamente metabolitos secundários, a planta *Castanea sativa* Mill. and e os fungos micorrízicos *Paxillus involutus* (Batsch) Fr. foram colocados em co-cultura por diferentes períodos (24h e 48h). A avaliação dos compostos fenólicos e voláteis, em ambas as espécies, foi avaliada antes do co-cultura e após o período??, o objetivo do presente trabalho foi (i) induzir a simbiose micorrízica do castanheiro com fungos compatíveis *in vitro*, e (ii) pesquisa de compostos fenólicos e voláteis envolvidos no processo micorrízico *in vitro*; (iii) análise da influência das raízes do hospedeiro no perfil desses compostos. Após o período de co-cultura, o cogumelo e as raízes de *C. sativa* foram analisados quanto à presença de compostos fenólicos e voláteis. Os compostos fenólicos foram avaliados por cromatografia líquida equipada com detector de arranjo de díodos e acoplado a detector de massa (LC-DAD-ESI / MSn). Os compostos voláteis, após extração por hidrodestilação, serão determinados por GC-MS (Cromatografia gasosa - espectrometria de massa).

Os resultados obtidos revelamos mesmos compostos fenólicos nas raízes controlo e nas raízes co-cultivados com fungo (24h e 48h). Em relação ao fungo (*P. involutus*), a

identificação foi muito difícil, mas encontramos fragmentos de compostos que deveriam estar presentes, a saber, ácidos (feniletanol).

Os cogumelos selvagens têm sido apontados como valiosos alimentos saudáveis, ricos em proteínas, vitaminas e minerais; uma fonte de compostos bioativos, como ácidos gordos insaturados ou mono / oligossacarídeos; e como tendo propriedades bioativas, como atividades antioxidantes, anti-inflamatórias ou antimicrobianas.

Por esse motivo, o outro objetivo deste trabalho foi demonstrar o potencial antioxidante (efeito sequestrador de radicais livres, poder redutor e inibidor da peroxidação lipídica), antimicrobiano (antibacteriano utilizando estirpes Gram-positivas e Gram-negativas e atividade antifúngica) e a ausência de toxicidade (células primárias de fígado suíno) dos extratos etanol / metanol, atividade anti-inflamatória, e contribuir com informações atualizadas sobre fontes naturais de compostos bioativos, caracterização química completa e possíveis aplicações.

Os resultados obtidos mostram que o extrato de todos os cogumelos silvestres apresentou atividade antioxidante, antimicrobiana, citotóxica e continha diferentes classes de várias moléculas bioativas, além de carboidratos, lípidos, proteínas e minerais. Para os resultados dos compostos fenólicos, as cinco amostras de cogumelos apresentaram 2 compostos fenólicos Ácido protocatecuico e ácido *p*-hidroxibenzóico.

Palavras-chave

Simbiose micorrízica, *Castanea sativa* ; *Paxillus Involutus;* cogumelos comestíveis; *Lactarius deliciosus; Boletus pinicola 2; Boletus pinicola 3 Tricholoma terreum; Tricholoma equestre*; metabolitos secundários, compostos fenólicos, compostos voláteis, compostos bioativos, atividades antioxidantes, anti-inflamatórias; antimicrobianas.

1. INTRODUCTION

Fungi

Fungi are eukaryotic organisms that include microorganisms such as yeasts and molds, as well as the mushrooms, fungi producing macrostructures to produce spores (sporophores or carpophores). These organisms are classified as a kingdom, separately from the other eukaryotic kingdoms, those being Plantae, Animalia, Protozoa, and Chromista. The existence of chitin in the cell walls of their cells is a key characteristic to place fungi in a different kingdom from plants, bacteria, and some protists[1].

Although mushrooms have a high biodiversity[2], part of an autonomous kingdom (Latin fungus = mushroom) [3], they are among the least exploited resources in the world. Fungi do not contain chlorophyll and are not photosynthetic organisms; they gain their energy and many of the nutrients to supply their biosynthetic pathways, through the degradation, symbiosis or parasitizing of plant and other organisms or matter[2], depending on being saprobiotic, symbiotic or parasitic in their trophic relations. However, it is estimated that this kingdom contains 2.2 to 3.8 million species[4], including 50,000 fungal species forming mycorrhizal associations[5].

During the present work, fungi collection was made during Autumn/Winter 2020 in different ecosystems and many different species from different trophic groups were collected and isolated *in vitro* for further studies to complete previous studies of the team work on chemical characterization and screening of mushrooms. This change in the original plan, was made taking into consideration the pandemic constraints that conditioned the accomplishment of the original plan as it was registered. These part of the work added more studies and training techniques to the thesis, including related parts of fungi culture and analysis.

The fungi collected and studied in the present work are listed and briefly described below: Two saprobiotic species (*Lepista nuda* and *Clitocybe odora*) and five mycorrhizal species *(Paxillus involutus, Boletus pinicola, Lactarius deliciosus, Tricholoma equestre, Tricholoma terreum)* were included.

Lepista nuda (Bulliard in 1790)

l.nuda (**Fig.1**), also known as the wood blewit and is a type of edible mushroom found in Europe and North America. It was first described by Pierre Bulliard in 1790, and for many years it was known as *Tricholoma nudum*.[6, 7].

It is a saprotrophic species, growing on decaying leaf litter. In the United Kingdom, it appears from September through to December and in Portugal from October to December [6, 7]. (The taxonomic classification of *Lepista nuda* is in the table 1 below).

Figure 1: *Lepista nuda* (real picture)

Clitocybe odora (Bull.)P. Kumn (aniseed toadstool)

The aniseed toadstool (**Fig.2**) is a blue-green mushroom that grows near deciduous and coniferous trees[7]. Saprobic and edible growing scattered or gregariously on hardwood litter in Europe and eastern North America on the debris of conifers or hardwoods[7].

C. odora is a tasty mushroom that can be fried with onions or used in risottos, soups, and a variety of other mushroom dishes. When these mushrooms are made into a sauce to serve with plaice, cod, or other white fish, the aniseed flavor is said to be especially strong.[7] (The taxonomic classification of *Clitocybe odora* is in the table 1 below).

Figure 2: *Clitocybe odora* (real picture)

Paxillus involutus (Batsch)Pers -(brown roll-rim)

P. involutus (Batsch) Pers (**Fig. 3**) is a model species for ecological or physiological studies of ectomycorrhizal agaricomycetes. Three to six groups or species of *paxillus involutus* linked to it have been ecologically and morphologically distinguished. Phylogenetic studies have revealed the existence of four species in Europe: *Paxillus ammoniavirescens, Paxillus obscurisporus, P. involutus,* and a fourth as yet not described species[6].

P. involutus is among the top four ectomycorrhizal organisms used as models for scientific studies that have contributed significantly to developments in understanding of the functioning of ectomycorrhizal symbiosis[6]. (The taxonomic classification of *Paxillus involutus* is in the table 1 below).

Figure 3: *Paxillus involutus* (real picture)

Boletus pinicola (Boletus pinophilus) (Pilat and Dermek)

Boletus pinophilus (Pilat and Dermek) (**Fig. 4**), commonly known as the pine bolete or pinewood king bolete, is a basidiomycete fungus of the genus Boletus found throughout Europe.[6]

The fungus grows in deciduous and coniferous forests and tree plantations, forming symbiotic ectomycorrhizal associations with living trees by enveloping the tree's underground roots with sheaths of fungal tissue. The fungus produces spore-bearing fruit bodies above ground in summer and autumn.[6]

The *B. pinophilus* is edible and may be used fresh, preserved, dried and cooked in a manner similar to that of other edible boletes, taking part of the group of boletes called the edulis group, referring to the edible high valued species of the genus Boletus[6]. (The taxonomic classification of *Boletus pinicola* is in the table 1 below).

Figure 4: Boletus pinicola[6]

Lactarius deliciosus (L.) Gray –Saffron Milkcap

This mushroom was described in 1753 by Carl Linnaeus, who gave it the binomial scientific name *Agaricus deliciosus* (**Fig. 5**). Synonyms of *Lactarius deliciosus* include *Agaricus deliciosus* L., and *Agaricus lactifluus* var *deliciosus* (L.) Pers.[7]

L. deliciosus, also known as saffron milk cap or red pine mushroom, is one of the most well-known members of the *Lactarius* genus in the Russulales order. *L. deliciosus* grows under conifers on acidic soils and forms a mycorrhizal relationship with its host tree.[7] (The taxonomic classification of *Lactarius deliciosus* is in the table 1 below).

Figure 5: *Lactarius deliciosus* (real picture)

Tricholoma equestre (L.) P. Kumm.

Tricholoma equestre or *Tricholoma flavovirens* (**Fig. 6**), also known as man on horseback or yellow knight, is a fungus of the genus *Tricholoma* that forms ectomycorrhiza with pine trees and was once commonly eaten.[7]

T. equestre has a long history of being collected from the wild as food because of its flavor. Both caps and stipes, which can be dried, frozen, or freshly cooked, are typically eaten in a variety of ways, including fried, boiled, soured, and pickled. *T. equestre* can be used to make a soup without blanching, according to some popular mushroom cookery books.[7] (The taxonomic classification of *Tricholoma equestre* is in the table 1 below).

Figure 6: *Tricholoma equestre* [7]

Tricholoma terreum (Agaricus terreus)

Tricholoma terreum (**Fig. 7**), also known as the dirty *Tricholoma* or grey knight, is a grey-capped mushroom belonging to the *Tricholoma* family.[7]

T. terreum is a grey *Tricholoma* species that can be confused with a number of other grey *Tricholoma* species. This large *Tricholoma* is mostly found in coniferous forests, but it can also be found under deciduous trees. It prefers calcareous soil to neutral soil. These gregarious mushrooms are seldom seen alone and are frequently seen in large groups.[7] (The taxonomic classification of *Tricholoma terreum* is in the table 1 below).

Figure 7: *Tricholoma terreum (real picture)*

Table1: Taxonomic classification of all mushrooms [6, 7]

MUSHROOM	*Kingdom*	*Division*	*Class*	*Order*	*Genus*	*Species*	*Author*
Lepista nuda	Fungi	Basidiomycota	Agaricomycetes	Boletales	Lepista	*L.nuda*	Pierre Bulliard in 1790
Clitocybe odora	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Clitocybe	*C.odora*	*Bull.)P.Kumn*
Paxillus involutus	Fungi	Basidiomycota	Agaricomycetes	Boletales	Paxillus	*P.involutus*	J.F. Gmel.Pers.
Boletus pinicola	Fungi	Basidiomycota	Agaricomycetes	Boletales	Boletus	*B.pinicola*	Pilat and Dermek (1973)
Lactarius deliciosus	Fungi	Basidiomycota	Agaricomycetes	Russulales	Lactarius	*L.deliciosus*	(L.) Gray, 1753)
Tricholoma equestre	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Tricholoma	*T.equestre*	(L.) P. Kumm
Tricholoma terreum	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Tricholoma	*T.terreum*	(L.) P. Kumm

Mycorrhization

Mycorrhizas are symbiotic associations between plants and fungi that colonize the cortex of their roots, being a beneficial association for both partners[8].

Mycorrhizae increase the surface area of the plant's root system because narrow hyphae can spread beyond the nutrient depletion zone. Hyphae are long extensions of the fungus, which can grow in small pores of the soil that allow access to phosphorus that otherwise would not be available to the plant. The beneficial effect on the plant is observed in the poor soils[9]. This symbiosis thus constitutes a distinctive system more efficient than the roots alone for the absorption and transfer of nutrients from the soil[10]. Mycorrhizae also function as a physical barrier against pathogens.

Mycorrhizal fungi are therefore associated with plant roots and promote nutrient absorption to the host plant. There are two main types of mycorrhizas: ectomycorrhizae and endomycorrhizae, which are the most common forms[9]. In the ecosystem, these soil inhabitants play a very important role, namely the recycling and, in particular, the biodegradation of organic matter, which plays a vital role since they are heterotrophic organisms, as well as their characteristic of forming reciprocal relationships in the form of a plant root molecular dialogue that ultimately results in symbiosis[10].This symbiosis is important in plant biology since its establishment improves the nutrient cycle, hence the host's nutritional status, affects growth, absorption of water and protection against root diseases[11].

Mycorrhizas are classified according to their structure, host families and symbiotic fungal taxa. There are seven specific types of mycorrhizal associations: *i) vesicular-arbuscular mycorrhizas (VAM),* widely known as *arbuscular mycorrhizas (AM); ii) ectomycorrhizas (ECM); iii) ectendomycorrhizas; iv) ericoid mycorrhizas; v) orchid mycorrhizas; vi) arbutoid mycorrhizas;* and *vii) monotropoid* mycorrhizas (in some classification systems, *arbutoid* and *monotropoid* are subtypes of *ectomycorrhiza* type*).* The most common association of all mycorrhizae is the *arbuscular mycorrhiza (AM),* followed by *ectomycorrhizas (ECM)* [12, 13].

The main mycorrhizal categories and subcategories are listed and described in Table 2.

Table 2 : Types and categories of the main mycorrhizal associations[12]

Categories	*Subcategories*	*Definition*
Endomycorrhizae		Do not form a dense sheath over the root. Instead, the fungal mycelium is embedded within the root tissue and enters the cells.
Ectomycorrhizae		Form an extensive dense sheath around the roots, called a mantle. Hyphae from the fungi extend from the mantle into the soil, which increases the surface area for water and mineral absorption.
	Cortical	Hartig net fungal hyphae colonize multiple cortex cell layers of short roots (most associations are in gymnosperms).
	Epidermal	Hartig net fungal hyphae are confined to the epidermal cell layer of short roots (occurs in angiosperms).
	Monotropoid	Exploitative epidermal ECM of myco-heterotrophic plants in the Ericales where individual hyphae penetrate epidermal cells.
	Arbutoid	ECM of autotrophic plants in the *Ericaceae* where multiple hyphae penetrate epidermal Hartig net cells.

Ecto- and endomycorrhizas, differ considerably in their structure and physiological relationships with symbionts [12].

In ectomycorrhizas, the fungus develops a sheath or mantle around the feeder roots. The mycelium penetrates between the cells of the root forming the Hartig net but not forming intracellular penetrations. About 3% of vascular plants, mainly forest trees (Fagaceae, Pinaceae, and Eucalyptus) form ectomycorrhizas. In endomycorrhizas, no sheath is formed, and the fungi colonize the root cortex both intercellularly and intracellularly. A few endomycorrhizal types are restricted to species of Orchidaceae ("orchid" mycorrhiza) [12].

Figure 8: represents the main differences between endo and ectomycorrhizae:

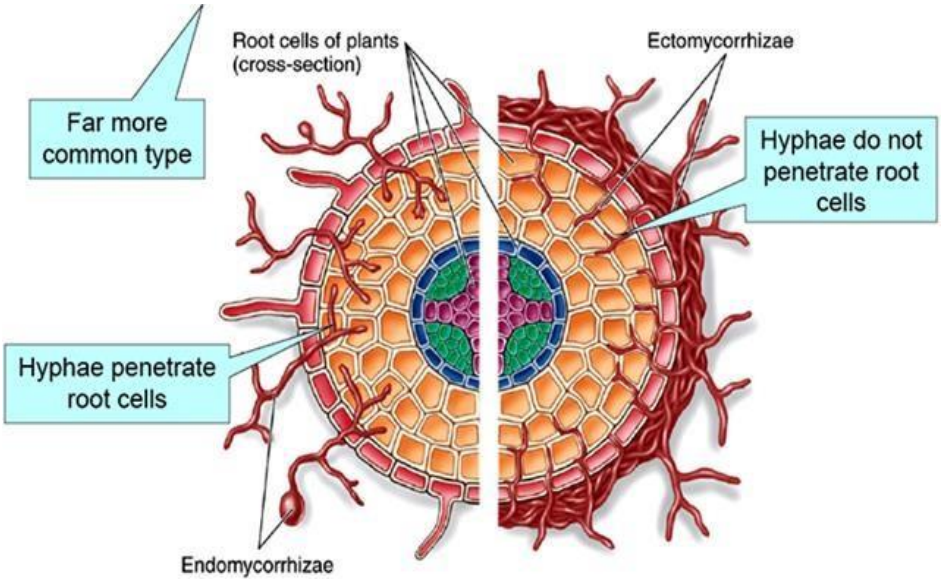

Figure 8: Endomycorrhizae and ectomycorrhizae: schematic sectional views. [13]

Ecological and biological function of mycorrhization

Ecological function

Fungi play a central role in many microbiological and ecological processes, influencing soil fertility, decomposition, cycling of minerals and organic matter, as well as plant health and nutrition[14]. The fungi play fundamental ecological roles as decomposers, or pathogens of plants and animals[14]. Fungi secrete several enzymes which can decompose cellulose, hemicelluloses and lignin mainly from plants. Other can solubilize soil compounds releasing mineral nutrients for the plants (**Fig. 9**)[15]. Therefore, they have a "mission" of great ecological importance[15].

Mycorrhizal fungi in particular connect their plant hosts to the nutrients required for their growth, facilitating the flow of energy-rich compounds required for nutrient mobilization. Simultaneously, provides channels for the translocation of mobilized products back to their hosts. In addition to increasing the nutrient absorptive surface area of their host plant roots, the extraradical mycelium of mycorrhizal fungi provides a direct pathway for translocation of photosynthetically derived carbon to microsites in the soil and a large surface area for interaction with other micro-organisms.[16]

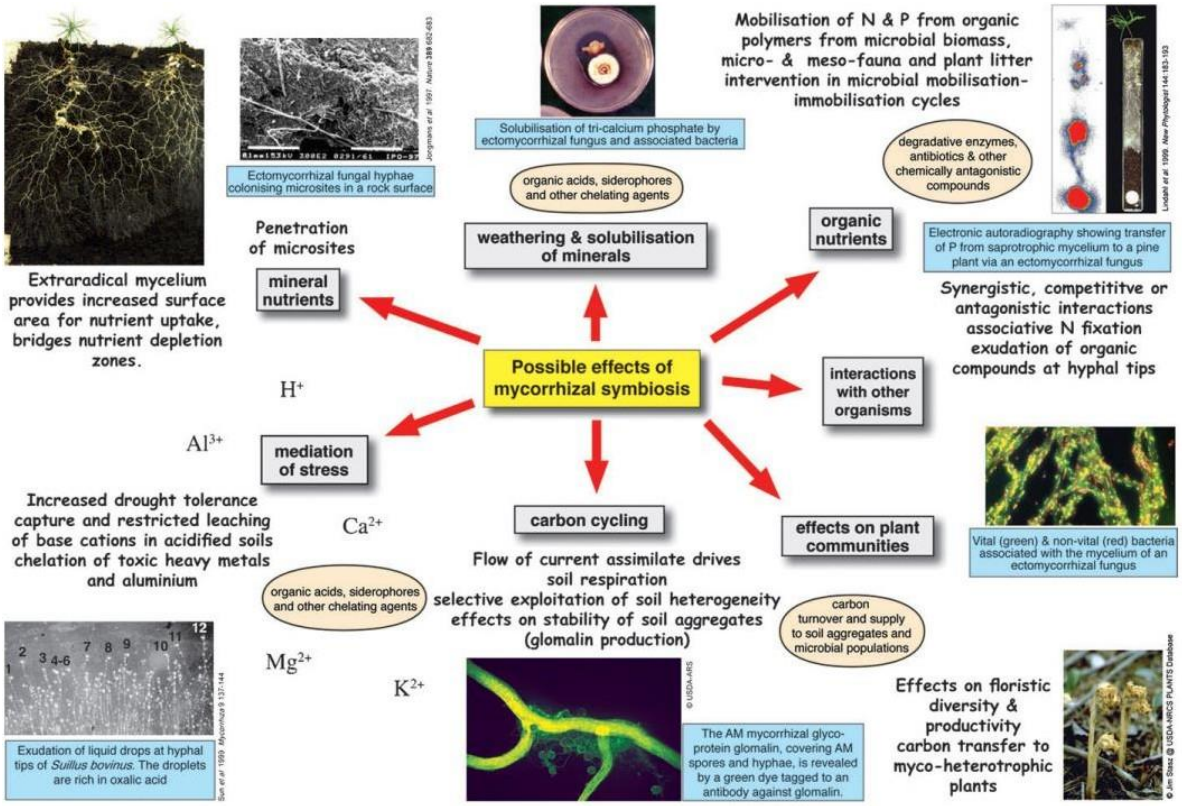

Figure 9: Schematic representation indicating de diversity of possible interactions involving the extraradical mycelia of mycorrhizal associations. [16]

Mycorrhizas must be faced in a multifunctional perspective, including the effects of mycorrhizal symbiosis on plant and microbial communities, and on ecosystem processes (**Fig. 9**). This includes mobilization of N and P from organic polymers, release of nutrients from mineral particles or rock surfaces via weathering, effects on carbon cycling, interactions with myco-heterotrophic plants, mediation of plant responses to stress factors such as drought, soil acidification, toxic metals, and plant pathogens, as well as a range of possible interactions with groups of other soil micro-organisms (**Fig. 10**).[16]

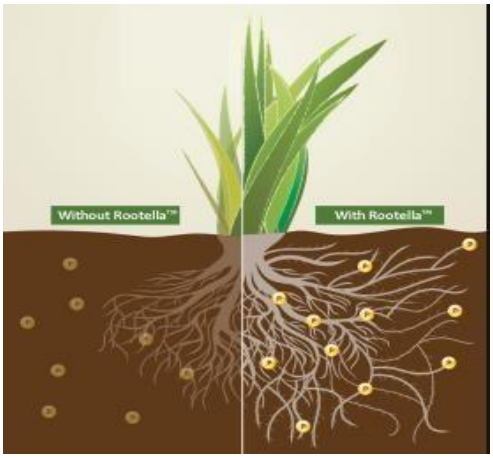

Figure 10: The difference between the plant with and without mycorrhza.[15]

Biological function

The mycorrhizal fungi deploy an array of biochemical and physiological mechanisms that act in a concerted manner to provide more tolerance to the host plant to salinity, some of the well-known mechanisms include improved: [14.15]

1. Fight off parasites
2. Superior water use efficiency and osmoprotection
3. Provide tolerance of many situations for the plants like (heat, salinity, and drought)
4. Nutrient uptake
5. Increase the photosynthetic efficiency of the plant
6. Preservation of cell ultra-structure
7. Strengthen the plant's adaptability to environmental changes.

Mycorrhizal symbiosis induces enhanced accumulation of anthocyanins, chlorophyll, carotenoids, total soluble phenolics, tocopherols, and various mineral nutrients.

Mycorrhization *in vitro*

- **Mycorrhization methods**

In vitro mycorrhization allow plants to contact mycorrhizal fungi grown *in vitro* under controlled conditions (temperature, photoperiod, nutrition…) and can be made by several axenic and non-axenic techniques. Axenic and non-axenic mycorrhizal methods mainly differ in the time and degree of infection[16].

- **Axenic Methods**

Among the characteristics of this method:

1. Interacting factors are eliminated.[16]
2. Carbon sources are provided to allow fungal growth before the infection sets in.[16]
3. Substrates are sterilized; this may change the efficiency and type of infection[17]
4. The axenic condition makes it possible to obtain a quality inoculum according to a simple and reproducible model. [16]

- **Non-Axenic Methods**

Non-axenic systems allow detailed studies of the root colonization by the fungus[16] and were performed to many purposes: [19.20]

1. Directly control cell permeability and the mechanism of fungus adhesion to the roots when mycorrhization takes place
2. Secretion of root polysaccharide hence the connection of fungi to the root epidermis.
3. The translocation of photosynthetic products to the root increases the concentration of carbon compounds in root exudates.

Mycorrhizas obtained by different methods of *in vitro* synthesis had mantles and Hartig nets with similar structures[21].

- ***In vitro* mycorrhization of micropropagated plants**

Micropropagated plants are adversely affected by water stress. The roots become adapted to a substrate with less available nutrients and an autotrophic condition. For this reason, the presence of mycorrhizal fungi is necessary to sucking up nutrients and water and passing them to the roots; acting as extensions of plant roots.[18]

Water stress can be responsible for the low survival of many micropropagated woody plant species during the acclimation process, and *Castanea sativa* Mill is one of these species[18], hence the interest of working on this plant (this plant, studied in the present work, is described below).

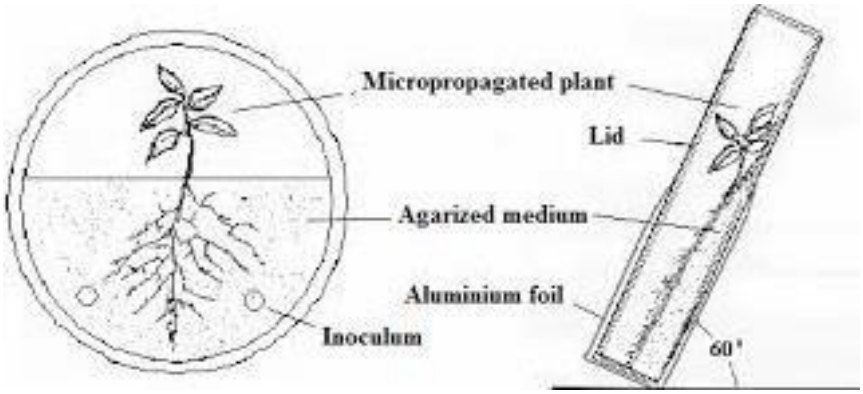

Figure 11: Axenic synthesis of micropropagated *Castanea sativa* Mill. mycorrhizas with *Pisolithust inctorius* (Pers.) Coker & Couch.[18]

Micropropagated plants development includes:

- High moisture environment
- Low photosynthetic efficiency due to in *vitro* lighting conditions
- Low lignifications levels
- Reduced functionality of the root systems.

The European chestnut is difficult to propagate by cuttings and show high heterogeneous seeds. So, the micropropagation of adult clones emerges as a methodology that aims to overcome this propagation difficulties[19].

Nowadays, some enterprises apply modern ***in vitro*** **propagation techniques** for the reproduction of plants whose sexual reproduction systems are not efficient or enough to meet market demand and the chestnut tree is among the plants produced by micropropagation of adult clones [19].

With these concepts in mind and considering that the laboratory of Biology and Biotechnology of the School of Agriculture of the Polytechnic Institute of Bragança has been working on the micropropagation of plants of high interest to the region, namely ***C. sativa***, these were the studied species and the technique adopted in the present work.

In vitro mycorrhization has several advantages:

(1) Artificial mycorrhization under controlled conditions can provide important information about the physiology of symbiosis.[16]

(2) Micropropagation and mycorrhization *in vitro* can be combining as a useful tool to improve the viability, performance, and survival capacities of the micropropagated plants.[20]

Castanea *sativa* Mill. (Chestnut tree)

Sweet chestnut

Castanea sativa Mill. belongs to the Fagaceae family and sub family Castaneoideae[21]. In Portugal, chestnuts are mainly cultivated for fruit production since timber has mostly lost its importance in recent years[22].China is the main chestnut producing country with more than 82% of world chestnut production and exports approximately 2% of its annual production[23].

Although chestnut orchards cover a large area in Portugal (the largest area of *C. sativa* in the European Union), and the Mediterranean climate features, have provided unique conditions for the remarkable evolutionary adaptation and divergence of life. The biodiversity of this region has been currently threatened by the habitat loss and degradation, provided by the pollution levels, drought, and species spread, over exploitation.[24, 25]

Figure 12: Castanea *sativa* Mill.[24]

Biological properties of the chestnut tree

C. sativa trees can be an important source of medicinal remedies. The leaves, bark, flowers, and spiny cases of the nuts are good astringents, which cause mucous membranes and skin to contract.[26]

They were, therefore used to control bleeding and aid in healing diarrhea. The leaves were used in teas to soothe irritated throats and in relieving symptoms of coughs and colds. The nuts are also reported in folk remedies for the treatment of fever, infections, inflammation, wounds, and stomach ailments.[27, 28]

Natural bioactive compounds

A bioactive compound is a substance that has a biological activity, related to its ability to regulate one or more metabolic processes, which results in the promotion of better health conditions and may belong to several biochemical groups.[29.30]

Phenolic compounds

Phenolic compounds are secondary metabolites produced as various antimicrobial substances by plants that hold at least one hydroxyl group in an aromatic ring. According to their basic skeleton, they can be classified into various groups, including phenolic acids, hydroxycinnamic acids, coumarins, naphthoquinones, xanthones, stilbenes and anthraquinones, flavonoids and isoflavonoids.[30]

These compounds are involved in plant growth, reproduction and plant defence mechanisms against fungal infections or ultraviolet radiation in plants. The importance of phenolic compounds stems from their flexible human health benefits, which include antioxidant, anti-inflammatory, antitumor and antimicrobial properties, among other results (**Fig. 13**)[31, 32].

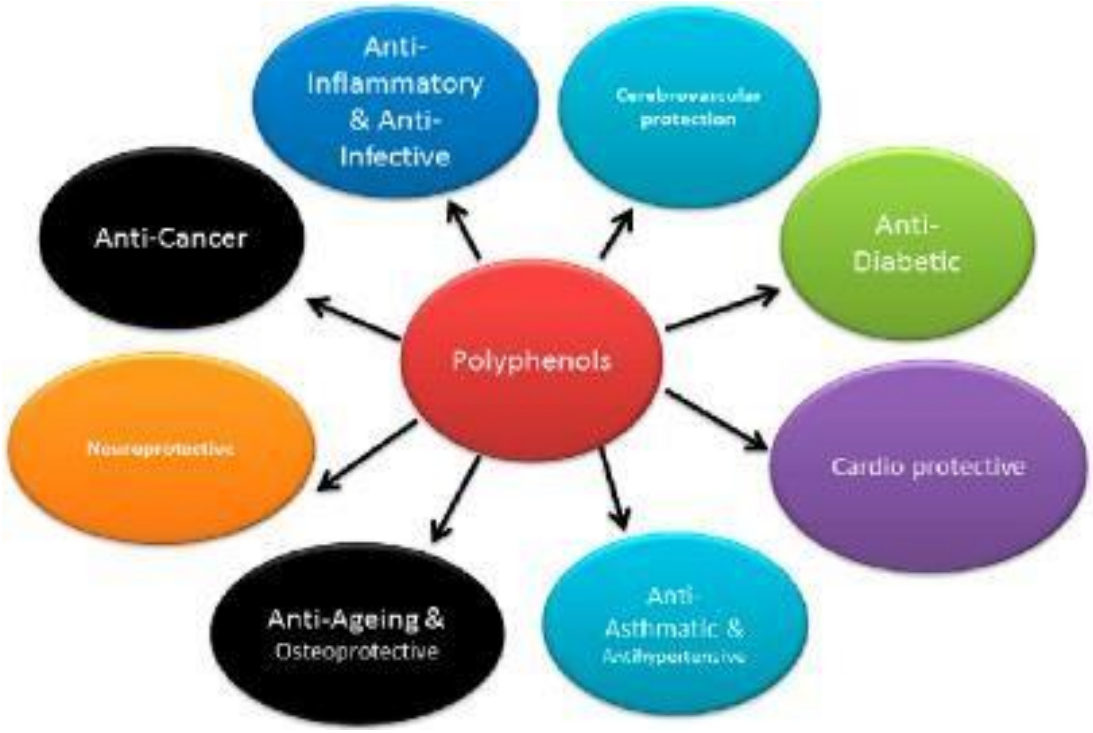

Figure 13: The different roles of phenolic compounds[34]

Flavonoids

Flavonoids, which are derivatives of simple phenols, are one of the largest groups of plant secondary metabolites. Flavonoids are responsible for the coloring of flowers and fruits [34, 35]. They can be located in various organs such as roots, stems, leaves, flowers and fruits. As other secondary metabolites, the synthesis of flavonoids increases after plant microbial infection, injury, decrease in temperature and deficiency of nutrients.[33, 34]

Tannins

Tannins are chemically defined as secondary compounds synthesized through plant secondary metabolism, or, for many authors, by special metabolism[35]. It's the astringent taste that repels insects, reptiles, birds, and superior animals.[36]

Tannins have been described as modulators in plant-herbivore interactions and protection agents against infection, with the main function as herbivore deterrents due to their acid taste and the property of precipitating proteins[37].

Other Organic acids

Other Organic acids are water-soluble organic compounds, such as formic and acetic acids, with acidic properties. They are classified as weak acids because, in aqueous solution,

they partially dissociate[30]. The acidity of an OA is usually characterized by at least one carboxyl group (COOH) and is determined primarily by the relative stability of the molecular conjugate base.[38]

Therefore, organic acids have been used as an important natural intervention to prevent food spoilage. They occur naturally in a variety of foods and, as a result of hydrolysis, biochemical metabolism, and microbial activity, are primarily found in fermented products. Even so, they are not considered nutrients, but they are responsible for supplying food with a distinctive taste.[38]

Volatile compounds

Volatile organic compounds (VOCs) are a major currency in plant communication where they mediate above- and below-ground interactions between plants, microorganisms, pollinators, herbivores, and they can be a crucial weapon in plant-plant competition[39].

VOCs produced by many organisms can have vital roles in inter organism's communication and also have the potential to act as characteristic biomarkers[40]**.** VOCs play a dominant role, being released by almost any kind of tissues and vegetation (trees, shrubs,grass, etc.) [41.42].

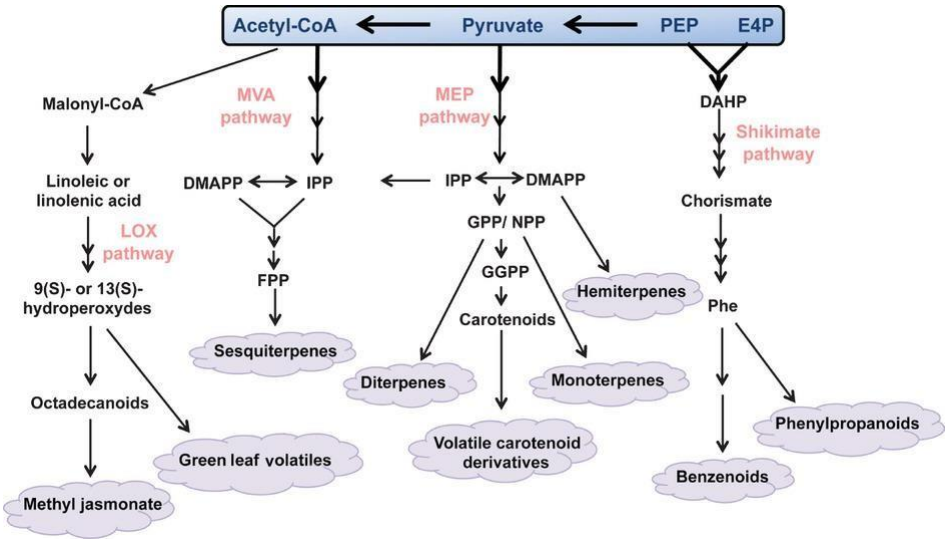

Figure 14: Overview of biosynthetic pathways leading to the emission of plant volatile organic compounds (VOCs). Precursors for plant VOCs originate from primary metabolism (represented in the blue box). The four major VOC biosynthetic pathways, namely the shikimate/phenylalanine, the mevalonic acid (MVA), the methylerythritol phosphate (MEP) and lipoxygenase (LOX) pathways lead

to the emission of benzenoids/phenylpropanoids, sesquiterpenes, monoterpenes, hemiterpenes, diterpenes, volatile carotenoid derivatives and methyl jasmonate/green leaf volatiles. Stacked arrows illustrate the involvement of multiple enzymatic reactions. VOCs are highlighted with a purple cloud as background. Abbreviations: DAHP, 3-deoxy-D-arabinoheptulosonate -7 phosphate; DMAPP, dimethylallyl pyrophosphate; E4P, erythrose 4-phosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranylpyrophosphate**.**[43]

Plants communicate with living things around them by emitting numerous different volatile compounds. They develop morphological and physiological defense mechanisms repulsing or attracting their enemies with these compounds[44]. Plants store these compounds produced for defense and release them in the form of volatile gases when needed. Plant volatile compounds include isoprene, terpene, fatty acid derivatives, alcohols, esters and volatile oils.[45]

Plant's emission of Volatile Organic Compounds (VOCs) is involved in a wide class of ecological functions, as VOCs play a crucial role in plants interactions with biotic and abiotic factors. The volatile products are very important because they are involved in vital ecological interactions such as:

- Indirect plant defense against insects (plant – pathogen interactions)[46]
- Pollinators' attraction[46]
- Plant-to-plant communication[47, 48]
- Thermo-tolerance and environmental stress adaptation[49]
- Defense from predators[50].

Vitamin E - Tocopherols

Vitamin E is a term widely used to refer to a family of chemically related compounds, namely tocopherols and tocotrienols, which share a chromanol head and isoprenic chain structure in general. Vitamin E consists of eight chemical compounds, namely tocopherol and tocotrienol of the alpha, beta, gamma and delta groups, which are synthesized from homogentisic acid by plants. Alpha-and gamma-tocopherols are the two major forms of the vitamin, with the relative proportions of these depending on the source.[51]

Vitamin E is the key lipid-soluble factor in the antioxidant protection mechanism of the cell and is obtained exclusively from the diet. For human health, it is extremely necessary.

In particular, it is well known for its antioxidant activity that prevents many diseases linked to oxidative stress.[52]

Fatty acids

Fatty acids, made up of carbon, hydrogen and oxygen, are basic organic compounds. Each fatty acid molecule has a carboxyl group (COOH) at one end (alpha) and a non-functional methyl group at the other (omega) (CH3).[53]

Carbohaydrate

Carbohydrates are the main source of energy for the body. They are the sugars, starches, and dietary fiber that occur in plant foods and dairy products. Carbohydrates are mainly found in plant foods. They also occur in dairy products in the form of a milk sugar called lactose.[53]

Bioactivity of mushrooms and related health effects

Mushrooms are widely consumed because of their exquisite and delicate flavor, as well as for their nutritional and chemical composition (high levels of minerals, protein, fiber and water, and low-fat contents) [54].

Beyond the nutritional characteristics, fungi have also been widely studied for their medicinal properties, particularly because of their richness in bioactive compounds with antioxidant, anti-inflammatory, and anticancer properties, among other bioactivities[55].

Bioactive compounds can be present in fungi as cell wall components such as polysaccharides (for example, β-glucans) and proteins, or as secondary metabolites such as phenolic, terpene, steroid and volatile compounds. The bioactive compounds' concentration and effectiveness are varied and depend on the substrate used, growth stage, age and freshness of the mushroom, and storage conditions[55].

It has been shown that the antioxidant activity of fungi is mainly due to the secondary metabolites present in these organisms, namely phenolic compounds, followed by tocopherols, ascorbic acid and carotenoids[56].

Since secondary metabolites such as phenolic and volatile compounds are abundant in the Kingdoms Plant and Fungi and given their function associated with organisms' defense

mechanisms, in this work, we chose to study this type of molecules (secondary metabolites) during the first stages of the mycorrhization process[57].

In addition to these compounds, many bioactive compounds have been identified in mushrooms. The following table (**table 3**) shows some of the compounds found in some mushrooms and their potential bioactivity.

Table 3:The potential bioactivity for some mushrooms

Mushroom Scientific name	***Bioactives compound***	***Bioactivity***	***Reference***
Boletus edulis	Polysaccharides	Anti-inflammatory	58
Boletus spp	24,6-trimethylacetophenone imine, glutamyl tryptophan, azatadine, lithocholic acid glycine conjugate	Antioxidant	59
Agaricus albertii Agaricus urinascens var. Excelle n Pleurotus eryngii	Phenolic acids, Organic acids, Tocopherols	Antioxidant	60
Cantharellus cibarius	Pyrogallol Phenolic acids	Anti-inflammatory	61
Lactarius deliciosus	Pyrogallol, Flavonoids	Anti-inflammatory	58
Lactarius rufus Pleurotus ostreatus,	Polysaccharides: (1,3), (1,6) b-D-glucans	Anti-inflammatory	62
Macrolepiota procera, Boletus impolitus Agaricus bisporus	Phenolic acids	Anti-inflammatory	60

Antioxidant activity

In normal or pathological cell metabolism, free radicals that have one or more unpaired electrons are formed. Three elements, namely oxygen, nitrogen and sulphur, can derive free radicals, creating ROS, RNS and RSS, respectively.[63]

Reactive oxygen species (ROS) are the most important class of reactive species originated in biological systems. ROS react easily with free radicals to become radicals themselves. ROS are various forms of activated oxygen, which include free radicals such as superoxide anion radicals and hydroxyl radicals, as well as non-free radical species (H_2O_2) and the singlet oxygen[64].

ROS, as well as non-free radical species (H2O2) and singlet oxygen, are different types of activated oxygen which include free radicals such as superoxide anion radicals and hydroxyl radicals. Excessive ROS generation, caused by multiple stimuli and exceeding the body's antioxidant ability, often leads to a variety of pathophysiological processes, such as inflammation, diabetes, genotoxicity, and cancer[64].

Antimicrobial activity

A broad range of pharmaceutical agents, including antibacterial, antifungal, antiviral and anti-parasitic medications, are included in the word antimicrobial.[65]

Bacterial tolerance may be inherent or innate, which means that some types of antimicrobial agents are immune to bacterial organisms. In addition, resistance can be gained, which means that originally susceptible populations become immune to the antibacterial agent and have been able to proliferate in its presence.[66]

Pathogenic fungi cause injury in humans, animals, crops, and other living organisms. Although fungal infections contribute substantively to human morbidity and mortality, and despite the need for efficient diagnostic tests and new medicines and vaccines, research on the pathophysiology of human fungal infections that cause disease falls short of other caused by other pathogens (such as the aforementioned bacteria),[67]

Dermatophytes, yeasts and moulds are the key pathogens responsible for fungal infections, being the most diseases caused by pathogenic fungi, superficial skin and nail

infections. Probably, around 25 per cent of the world's population is affected by skin mycosis.[68, 69]

Cytotoxicity activity

Natural products derived from biological species have proved to be beneficial and cost-effective sources of new drug entities. Numerous vaccines and other medical products have been produced as a result of these natural products [70]. Some species, such as mushrooms, are considered to be toxic to humans and animals as a result of their natural survival and protection mechanism to survive in the environment.

In this context, cytotoxicity testing is crucial for evaluating the potential of biologically active compounds isolated from these species [71]. A cytotoxicity analysis is considered a standard of practice in the pharmaceutical and cosmetic industries to assess the level of toxicity of any new medications or products.

These toxicity findings, whether minimal or nontoxic, are essential to the creation of new discoveries. In this regard, cellular toxicity research is important for introducing new varieties of edible species that are both safe for human consumption and have the potential to be used for therapeutic purposes.

Anti-inflammatory activity

Inflammation is now widely accepted as a factor in the progression of a variety of chronic diseases, including arteriosclerosis, obesity, diabetes, neurodegenerative diseases, and cancer. The problem of treating chronic inflammatory disorders has yet to be solved, and new and safe anti-inflammatory preventive and therapeutic compounds are urgently needed.[72]

Medicinal mushrooms have long been used in Asian countries to control and treat a range of ailments. Edible mushrooms, on the other hand, have recently gained popularity as a functional food due to their antimutagenic, antitumor, antiviral, anti-thrombotic, hypocholesterolemic, hypolipidemic, and anti-oxidant properties. Among all of these beneficial properties, the immunomodulatory activity of some fungal compounds such as polysaccharides, especially -glucans was highlighted. Owing to the existence of bioactive compounds, some studies have indicated that several mushroom species may have anti-inflammatory properties based on their ability to reduce inflammatory mediator output.

Polysaccharides, phenolic compounds, proteins, fatty acids, and other bioactive metabolites have been isolated from mushrooms and implicated as responsible for anti-inflammatory behavior.[72]

1.7. Future Trends

Mushrooms are functional food and are a source of biologically valuable components that offer great therapeutic potential to prevent and control several diseases. Many mushroom-derived bioactive compounds, both cellular components and secondary metabolites, have been isolated. Some studies about mushrooms' bioactivity were assayed using crude mushroom extracts or a mixture of mushroom metabolites. These studies require the isolation and identification of the bioactive compounds to determine each compound's bioactive effect. The optimization of submerged culture conditions for mycelia growth and strain improvement by genetic manipulation is crucial to overproduce the desired compound. Further research and clinical trials have to be carried out to validate that mushrooms are a bioactive source with the medicinal application.

2. OBJECTIVES

The present work suffered a few adaptations due to the pandemic situation, since part of the laboratory work was not possible to be started on time during the closure of presential activities from March 2020 to June 2020. The remaining time conditioned the longer trials, namely the plant micropropagation process to produce the necessary number of rooted plants.

Meanwhile the viability of some fungi *in vitro* decreased, and it was necessary to return to wild fungi collection in autumn 2020, to isolate new species and strains. Facing these difficulties, we readapted the objectives including the collection and *in vitro* isolation of fungi, growth rates of isolates in different media and, the chemical study of some of the species collected, following the chemical characterization that we have being doing along time to screen the wild native species for important compounds and bioactive activities.

The chemical characterization of the wild mushrooms was made in the species that were not yet studied previously or in species which chemical and bioactive profile needed to be completed according to the chemical and nutritional inventory of Portuguese wild edible mushrooms in different habitats that is being performed at CIMO-IPB group (http://esa.ipb.pt/biochemcore/index.php/studied-mushrooms). [73]

- **The main objective of this master´s thesis was to:**

Screen for phenolic and volatile compounds involved in the *in vitro* mycorrhizal process of *Castanea sativa Mill* with compatible mycorrhizal fungi, namely *P. involutus* (Batsch) Fr. Following the intermediate steps:

- Production of chestnut tree plants *in vitro*.
- Production of mycorrhizal fungi mycelium *in vitro*.
- Mycorrhizal induction through co-culture of plants and fungi *in vitro*.
- Analysis of phenolic compounds and volatiles compounds composition of Mycorrhizal and Non Mycorrhizal roots and fungi (*Pi*) in two stages / time points of the mycorrhization process.

Therefore, it was intended to evaluate the potential production of compounds related to oxidative stress during mycorrhizal induction processes, either in mycorrhizal fungi and the roots of *Castanea sativa* in co-culture for 24h and 48h. Since volatile compounds act as

chemical signaling molecules, their formation during this recognition process was also intended to be screened.

- **Additional objectives of this master´s thesis introduced following reorganization of the plan according to the chemical and nutritional inventory of Portuguese wild edible mushrooms were**:
- *In vitro* isolation and culture of wild mycorrhizal mushroom (*Tricholoma equestre, Tricholoma terreum, Boletus pinicola* (strains 1, 2 and 3) *and Lactarius deliciosus*) and two saprobic species *Clitocybe odora* and *Lepista nuda.*.
- Analysis of natural bioactive compounds of wild mycorrhizal mushroom (phenolic compounds, volatiles compounds and organic acids).
- Analysis of the bioactivity of wild mycorrhizal mushroom (antioxidant, antimicrobial, anti-inflamatory and cytotoxicity activities) of *Tricholoma equestre, Tricholoma terreum, Lactarius deliciosus* and *Boletus pinicola.*
- Analysis of nutritional value of *Tricholoma terreum* (total fat, fatty acid, proteins, ash, soluble sugars).

3. MATERIALS AND METHODS

To achieve the objectives described above, several methods were used: Chestnut plants were produced using *in vitro* techniques, and after rooting, the fungus *P. involutus* was co-cultivated with the roots for 24h and 48h. After the co-culture period, roots and fungus were harvested, and their phenolic and volatile compounds profiles were extracted, and phenolic compounds evaluated (volatiles were extracted but not yet evaluated). Afterwards, the obtained profiles were compared with the profiles of roots and fungus grown alone.

To achieve the remaining objectives of our work, 5 different edible mycorrhizal mushroom species were used (*Tricholoma equestre, Tricholoma terreum, Lactarius deliciosus* and *Boletus pinicola* (2, 3) and their extracts (mainly methanolic) were used to evaluate their bioactive potential, namely antioxidant (TBARS, Oxhilia), antimicrobial (by the determination of **MBS** (Minimal Bactericial Concentration), and **MIC** (Minimal Inhibitory Concentration), anti-inflammatory properties (by the method of *Taofiq et al* 2015), cytotoxicity activity (by the determination of the tumour and non tumour cell lines) and also by the evaluation of the nutritional value of *T. terreum* (total fat, fatty acid, proteins, ash, soluble sugars).

Work 1:

Production of chestnut tree plants and mycorrhizal fungi mycelium *in vitro*: [16]

Chestnut tree multiplication and rooting *in vitro*

C*astanea sativa* Mill. (obtained from previous cultures maintained in the Biology and Biotechnology Laboratory of the School of Agriculture of the Polytechnic Institute of Bragança, Portugal) were obtained through a process that consisted of several steps here described but, applied here only from the establishment step on:

Axillary buds were sterilized with sodium hypochlorite 5% and two drops of Tween 80 under agitation for 15 min. After several washes with sterile water, the buds were poured in ethanol for 5 min, washed again with sterilized water, and finally inoculated *in vitro* in Murashige and Skoog (MS) modified medium in test tubes. The test tubes were kept in the dark at 25 ºC for 48 h, and then exposed to light for day and night photoperiods (16h/8h),

respectively, in a culture chamber with Daylight lamps. After establishment, the plantlets were transferred to multiplication media for several multiplication cycles and rooted *in vitro* to reach the ideal size and number. This process is being done continuously for plant multiplication, and it was the procedure used to produce the plants used in the present work.

Multiplication step

The plantlets were sub-cultured into new flasks with a solid culture medium with the purpose of multiplication and elongation of the shoots (**Fig. 15**). The MS (Murashige and Skoog)[57] medium pH 5.5 was used(NH_4NO_3 825.0 mg/l; KNO_3 950.0 mg/l; $CaCL_2 2H_2O$ 440.0 mg/l; $MgSO_4.7H_2O$ 370.0 mg/l; KH_2PO_4 170.0 mg/l; Fe Na EDTA 80.0 mg/l; myo-inositol100.0 mg/l; KI 0.83 mg/l; H_3BO_3 6.2 mg/l; $MaSO_4.4H_2O$ 22.3 mg/l; $ZnSO_4.7H_2O$ 8.6 mg/l; $Na_2MoO_4.2H_2O$ 0.25 mg/l; $CuSO_4.5H_2O$ 0.025mg/l; $CoCL_2.6H_2O$ 0.025 mg/l; nicotinic acid 0.5 mg/l; pyridoxine HCL 0.5mg/l; tiamine HCL 0.1 mg/l; glycine 20mg/l; sucrose 20.0 g/l; agar8.0 g/l)[63].

The explants were maintained in the *in vitro* culture chamber of the above-mentioned laboratory, at 23 °C / 18 °C thermoperiod and 16 h / 8h photoperiods.

Figure 15: Plantlets sub-cultured into new flasks with MS solid culture medium

After 6 weeks of growth, plantlets with 5 cm long or more were (**Fig. 16**) submitted to a rooting induction on MS medium with 3g/L activated charcoal, after dipping of plants in a concentrated IBA solution (5mg/mL) for 10 minutes. The shoots with less than 5 cm were multiplied and sub-cultured into new MS medium to continue their proliferation.

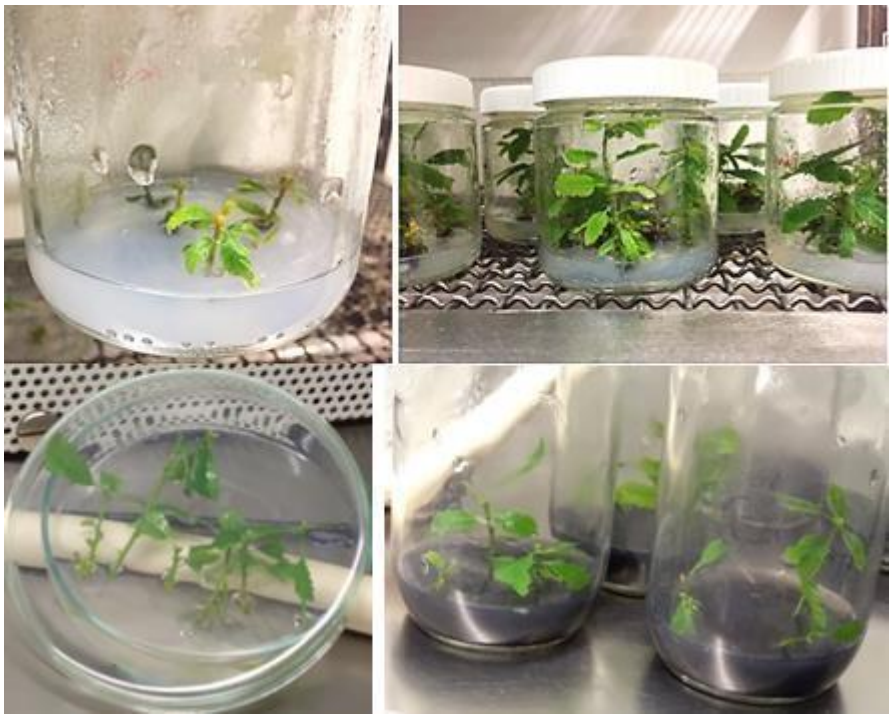

Figure 16: Chestnut plants *in vitro* multiplication and rooting steps.

In vitro production of *Paxillus involutus*.

P. involutus (Batsch) Fr. mycelium was obtained from previous cultures maintained in the laboratory of Biology and Biotechnology of the School of Agriculture from the Polytechnic Institute of Bragança, Portugal.[54, 55]

The mycelia were maintained in an incubator of the above-mentioned laboratory at 25°C. The medium used was Melin-Norkans medium (MMN) pH 6.3 (NaCl 0.025 g/l; $(NH_4)2HPO_4$ 0.25 g/l; KH_2PO_4 0.50 g/l; $FeCl_3$ 0.005 g/l; $CaCl_2$ 0.050 g/l; $MgSO_4.7H_2O$ 0.15 g/l; thiamine 100 µg/l; malt extract 5 g/l; casaminoacids 1 g/l; glucose 10 g/l; and agar 20 g/l (in solid medium)) .

The mycelium isolation from mushrooms was carried out under sterile conditions (laminar flow hood), in Petri dishes (9 cm in diameter) with 10 ml of solid MMN medium. After isolation, the sub-culture and mycelia mass production was made in solid medium as above. (**Fig. 17**).

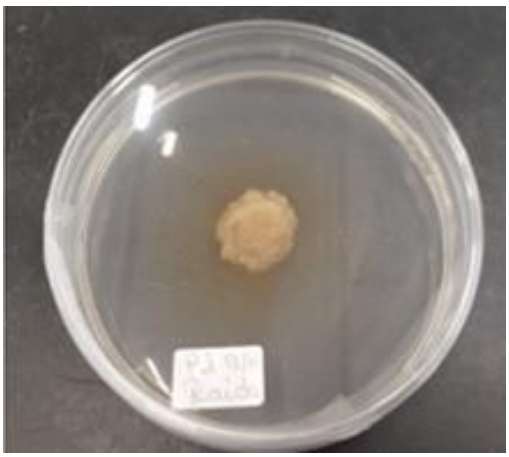

Figure 17: Mycelia of *P. involutus* obtained and maintained by *in vitro* culture (with MMN medium)

For the mass production of mycelia of *P. involutus* in solid medium, a thin layer of sterilized cellophane was used. 500 slices of cellophane paper were cut, placed on the Petri dishes, and then distilled water was added. Petri dishes were placed in the microwave to boil water, which was renewed (5 times). In the end, they were placed in the autoclave to remove the fungicide.

Sterile cellophane was placed on the medium surface, above which the fungus was inoculated, to facilitate the recovery of the mycelium after growth[56]. (**Fig. 18**).

Figure 18: The sterile cellophane placement in the Petri dishes; Inoculation of the fungi in solid medium

After inoculation, the plate's surface starts to be completely covered with mycelium to obtain higher yields for the chemical analyses. (**Fig. 19**).

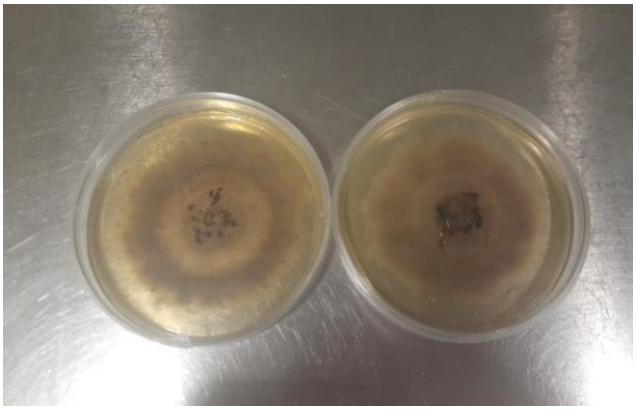

Figure 19: Mass production of fungi in solid medium

In vitro co-culture of *C. sativa* and *P. involutus*

Five to six weeks after rooting, roots were excised and co-cultured with the fungi mycelia in a solid co – culture system *in vitro*. Part of the roots used for the trials were supplied by DEIFIL to whom we thank for the material.

Fungi mycelium was cultured in Petri dishes (12 cm diameter) with incomplete MMN (without malt and casaminoacids) and maintained for 20 days in the dark.

The induction of the mycorrhizal symbiosis was carried out after the recovery of the roots, placing them in the Petri dishes containing the well-developed inoculum of the fungus. The co-cultures were maintained for 24h and 48 h.

After the co-culture periods, the mycelia were recovered from the solid culture medium. Afterwards, the recovered mycelia and roots were weighted to obtain the fresh weight, frozen and lyophilized, calculating the samples' dry weight.

Plant roots and fungi not co-cultured were weighed, frozen and lyophilized as controls of the trials.

After the established periods, the culture medium, roots and mycelia were recovered separately to assess the parts of the mycorrhizal system richer in the survey compounds. (**Fig. 20 to 23**).

Figure 20: Roots co-cultured in the MMN medium, weighing and then prepared to freeze.

Figure 21: Roots and *P. involutus* co-cultured together *in vitro* systems for different periods (24h and 48h), then the roots recovered separately.

Figure 22: Roots recovered separately *in vitro* systems for different periods (24h and 48h).

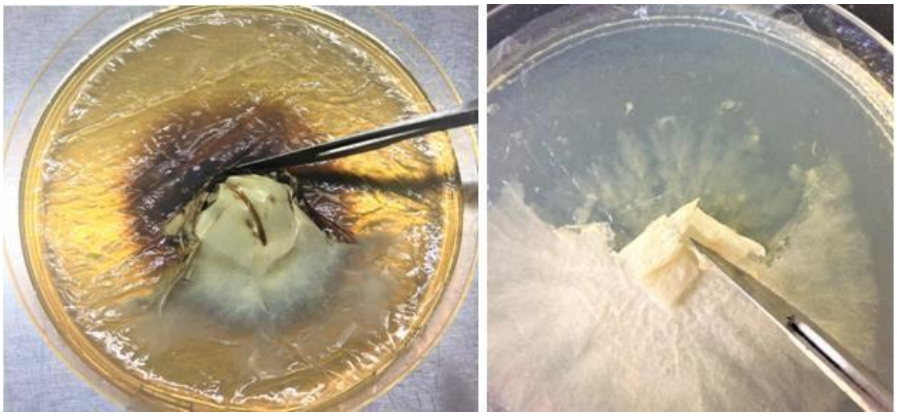

Figure 23: Fungi recovered separately *in vitro* systems for different periods (24h and 48h).

The recovered mycelia and roots were weighted to obtain the fresh weight (Fw), frozen and lyophilized, matching the dry weight (DW). The lyophilized samples were then protected from light, until further analysis.

Analysis of phenolic compounds composition

Extraction procedure

The techniques to extract phenolics employ solvents, either organic or inorganic. Several parameters may influence the yield of phenolics, including extraction time, temperature, solvent-to-sample ratio, the number of repeat extractions of the sample, as well as solvent type.

All the samples of the fungi and the roots were lyophilized and ground into powder, After having powdered roots and mushrooms (roots and fungi controls, fungi and roots co-culture together 24 h and 48h), 1g of each lyophilized powdered sample were extracted with 30 mL of methanol under magnetic stirring for 1h at room temperature (**Fig. 24**). Then the residue was re-extracted maintaining the same operational conditions. The combined extracts were evaporated at 40 ºC in a rotary evaporator (Büchi R-210, Flawil, Switzerland) to remove the alcohol. Afterwards, the samples were frozen and further lyophilized. The lyophilized extracts were re-dissolved at a concentration of 10 mg/mL in ethanol/water (80:20, v/v) and filter for an HPLC analysis.

Figure 24: Methanolic extract of the mushrooms and the roots

3.3.2. Chromatographic analysis

The extracts were analysed using a Dionex Ultimate 3000 UPLC instrument (ThermoScientific, San Jose, CA, USA) equipped with a diode-array detector and coupled to a mass detector (LC-DAD-ESI/MSn). The chromatographic system consisted of a quaternary pump, an autosampler maintained at 5ºC, a degasser, a photodiode-array detector, an

automatic thermostatic column compartment. The chromatographic separation was carried out on aWaters Spherisorb S3 ODS-2 C18, (3 μm, 4.6 mm × 150 mm, Waters, Milford, MA, USA) column thermostatted at 35 °C. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic 15% for 5 min, 15% B to 20% B over 5 min, 20-25% B over 10 min, 25-35% B over 10 min, 35-50% for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min.

Double online detection was carried out in the DAD using 280, 330 and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet. The mass spectrometer was operated in negative ion mode using Linear Ion Trap LTQ XL mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an ESI source. Typical ESI conditions were nitrogen sheath gas 50 psi, spray voltage 5 kV, source temperature 325 °C, capillary voltage -20 V, and the tube lens offset was kept at voltage of -66 V. The full scan covered the mass range from m/z 100 to 1500. The collision energy used was 35 (arbitrary units). Data acquisition was carried out with Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA).

The phenolic compounds were identified by comparing their retention times, UV-vis and mass spectra with those obtained with standard compounds, when available. Otherwise, compounds were tentatively identified comparing the obtained information with available data reported in the literature. For quantitative analysis, a calibration curve for each available phenolic standard (The same calibration curve for the roots control and roots co-culture was used: ellagic acid and y = 26719x – 317255), for the fungus *paxillus involutus* we do not have standard calibration curves to quantify these types of compounds, for the edible mushrooms the standard calibration curves was used is procatechuic acid (y=214168x+27102) and p-hydroxybenzoic acid (y=208604x+173056)), constructed based on the UV-Vis signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of another compound from the same phenolic group. The results were expressed as mg per g of extract.

Analysis of volatile compounds composition

Extraction of the essential oils

The extraction of essential oils by hydrodistillation will be performed using a Clevenger apparatus and by L-N.

Hydrodistillation is a traditional method for extraction of bioactive compounds, mainly essential oils from plants. Organic solvents are not involved, and it can be performed before dehydration of plant materials. Hydrodistillation is made in the Clevenger apparatus, which has a long tube, at the end of which the vapor is condensed and recovered. The volatile oil is removed from the top of the hydrolate (**Fig. 25**) and by L-N.

Figure 25: Clevenger apparatus

Chemical Analysis of the essential oils:

A fraction of each essential oil will be analyzed concerning the volatile composition by GC and GC-MS.

Gas chromatography (GC):

Gas chromatographic analyses must be performed using a Perkin Elmer Autosystem XL (Perkin Elmer, Shelton, Connecticut, USA) gas chromatograph or similar equipped with two flame ionization detectors (FIDs), a data handling system and a vaporizing injector port into which two columns of different polarities are installed: The samples are injected using split sampling technique. The percentage composition of the oils will be computed by the normalization method from the GC peak areas, calculated as mean values of two injections from each essential oil, without using correction factors.

Gas chromatography -mass spectrometry (GC-MS):

The GC-MS unit will be a Perkin. Elmer Autosystem XL (gas chromatograph, equipped with DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm) (J & W Scientific, Inc.), and interfaced with a Perkin-Elmer Turbomass spectrometer (software version 4.1, Perkin Elmer, Shelton, Connecticut, USA. The identity of the components will be assigned by comparison of their retention indices, relative to C9-C17 n-alkane indices and GC-MS spectra from a homemade library, constructed based on the analyses of reference oils, laboratory-synthesized components and commercial available standards.

Work 2:

Wild mycorrhizal mushroom collection and *in vitro* culture establishment:

Collecting mushrooms in the forest

Mushrooms were harvested from different habitats in Bragança in November 2020. The species here listed were isolated *in vitro* and the remaining carpophores used for different assays of chemical and biochemical characterization **(table 4)** in order to contribute to the chemical and nutritional inventory of Portuguese wild edible mushrooms initiated and developed at Mountain Research Center – CIMO-IPB (**Fig. 26)**.

Figure 26: Mushrooms were harvested from different habitats in Bragança: (*Lepista nuda, Clitocybe odora, Tricholoma terreum, P. involutus, Lactarius deliciosus, Boletus pinicola* and other).

As can be verified in table 4 we attempted to isolate the species *L. nuda, C. odora, T. terreum, P. involutus, L. deliciosus, B. pinicola* and in case of *B. pinicola* three carpophores were used for isolation constituting three strains of the species. Since these carpophores had a considerable biomass, it was possible to make most of the trials separately for each of them and to compare the differences between genotypes.

Table 4 : Trials performed with the harvested wild mushrooms listed

Mushroom species	***In vitro isolation***	***In vitro growth***	***Nutritional***	***Anti oxidant***	***Anti microbial***	***Anti-inflamatory***	***Cyto toxicity***	***Volatiles compounds***	***Phenolic compounds***	***Organic acids***
Boletus pinicola 1	X	X						X		
Boletus pinicola 2	X	X		X	X	X	X	X	X	X
Boletus pinicola 3	X	X		X	X	X	X	X	X	X
Paxillus involutus	X	X						X	X	
Lactarius deliciosus	X	X		X	X	X	X	X	X	X
richoloma terreum	X	X	X	X	X	X	X	X	X	X
richoloma equestre	X	X		X	X	X	X	X	X	X
Lepista nuda	X	X						X		
Clitocybe odora	X	X						X		

NOTE: For *B.pinicola, three* carpophores were used for isolation constituting three strains of the species (*B.pinicola* 1, 2 and 3).

3.5.2. Isolation of mycorrhizal fungi *in vitro*:

Isolation of mycorrhizal fungi *in vitro* is done under sterile conditions. It consists in cutting small pieces of the fresh mushrooms and placing them in Petri dishes, which are filled with culture medium MMN (**Fig. 27**), to ensure the growth of the mycelium.

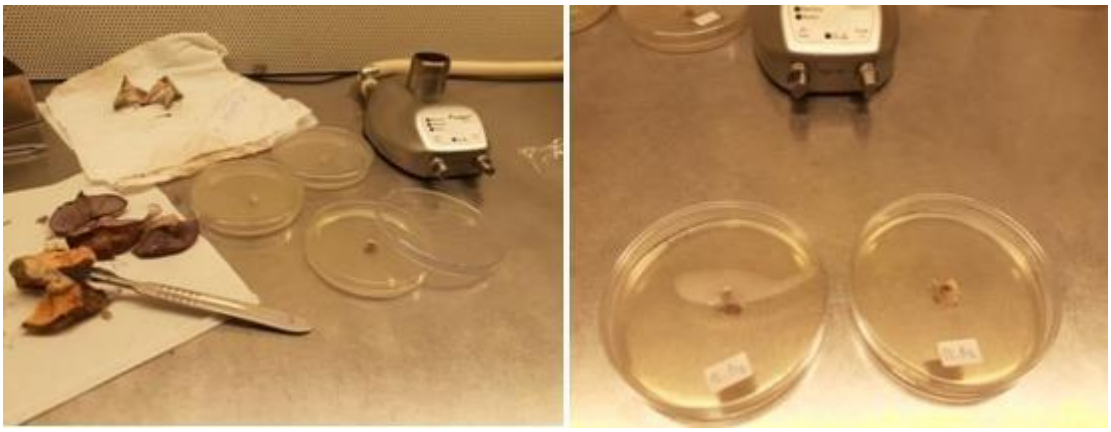

Figure 27: Isolation of wild fungi *in vitro*

Growth of wild mushrooms in two different types of culture medium

To compare the growth of the different wild fungi in 2 different types of culture medium (MMN: *Melin-Norkans medium* and PDA: *Potato dextrose agar*), to optimize the growth conditions for each species, because there are some fungi that grow better in the MMN medium while others in the PDA medium. After growth in the isolation medium, 10 Petri dishes of each medium were prepared for each species of fungus and the growth was followed in a week basis, measuring the two main diameters of the fungi growth.

Comparison of the growth of different fungi in two types of culture medium (PDAand MMN)

As shown in the pictures below, there are some fungi that grow better in the MMN medium and other in the PDA medium **(Fig. 28)**, So the fragment of the same mushroom were put it in two different culture medium and leave them for 3 weeks, and in each week, the diameter of mycelium growth were marked in the Petri dishes and then the average growth was calculated. (**Fig. 29**)

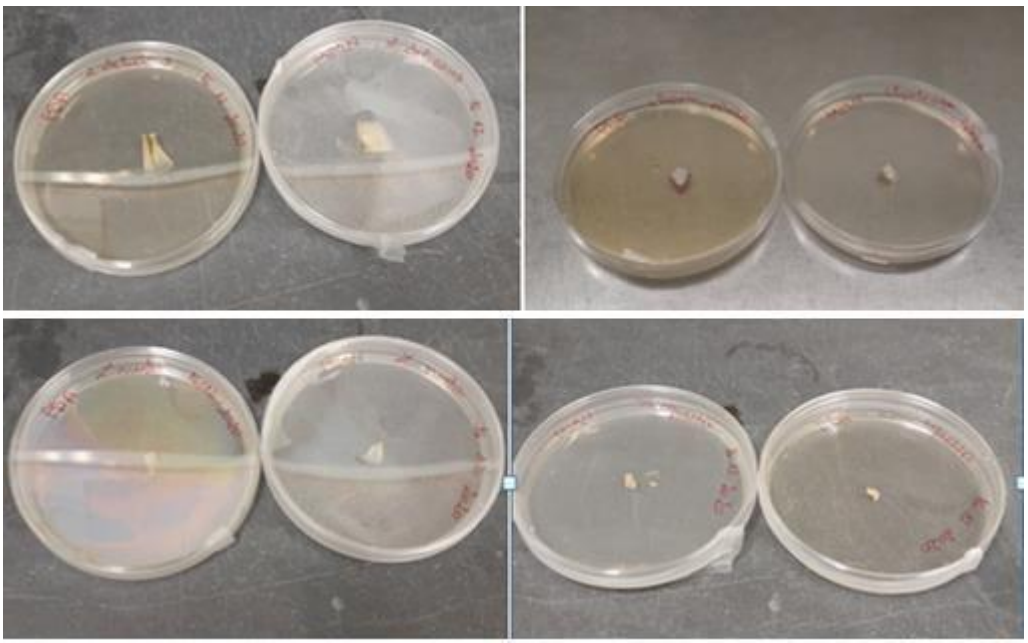

Figure 28: Inoculation of *L.deliciosus, C. odora ,L. nuda* and *T. equestre* in two different culture media (MMN and PDA)

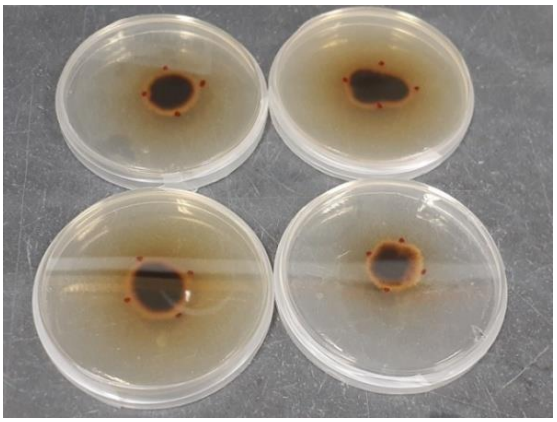

Figure 29: Growth diameter marking for fungi

Analysis of phenolic compounds composition

After isolation, the remaining carpophores were used for chemical and biochemical analysis. All the samples of the fungi were lyophilized and ground into powder: *T. terreum* (38.08g); *L. deliciosus* (24.48g); *B. pinicola*2 (15.20g), *B. pinicola* 3 (9.16g), *T. equestre* (20.02g). The weight of each mushroom after the freeze-drying was: *T. terreum* (28.7g); *L. deliciosus* (11.72 g); *B. pinicola*2 (9.8g), *B. pinicola*3 (5.50g), *T. equestre* (11.5g) (**Fig. 30**).

Figure 30: Mushroom's ground into powder

After having powdered mushrooms, 2 g of each lyophilized powdered sample were extracted with 50 mL of methanol under magnetic stirring for 1h at room temperature (**Fig. 31**). Then the residue was re-extracted maintaining the same operational conditions. The combined extracts were evaporated at 40ºC in a rotary evaporator (Büchi R-210, Flawil, Switzerland) to remove the alcohol. Afterwards, the samples were frozen and further lyophilized.

Figure 31: Methanolic extraction of the mushrooms

The lyophilized extracts were re-dissolved at a concentration of 10 mg/mL (C=m/v) in ethanol/water (80:20, v/v) for the antioxidant activity assay, in DMSO (10 mg/mL) for analysis of antimicrobial activity and in distilled water at a concentration of 8 mg/mL to evaluate the cytotoxic activity. Subsequently, these solutions were diluted successively in order to obtain the concentrations necessary to perform the experimental work.

Organic acids profile

Samples (approximately 1g) were extracted by stirring with 25 mL of meta-phosphoric acid (4.5%) for 45 min and subsequently filtered through Whatman No. 4 paper into a 20 ml test tube for 20 min, the analysis was performed using ultra fast liquid chromatography (UFLC) couple to photodiode array detector (PDA).

Figure 32: Extraction of organic acids

Antioxidant activity

The antioxidant properties of the mushroom species were assessed on the basis of their decreasing strength, scavenging activity of free radicals and ability to inhibit lipid peroxidation. There is no single assay that shows exactly the mechanism of action in a complex system of all radical sources or all antioxidants[74]. That is why two distinct approaches have been carried out.

TBARS formation inhibition

In this case, the lipid peroxidation can be determined by oxidation products that react with thiobarbituric acid (TBA) giving rise to pink compounds that are known as thiobarbituric acid reactive species (TBARS). One of the products commonly used as a biomarker of lipid peroxidation is malodialdehyde (MDA), which, associated with TBA in the presence of H^+ ions, form a chromogen (MDA-TBA). In this methodology, the oxidation of a lipid-rich preparation is induced by addition of a metallic ion (iron or copper), and the extension of the reaction with TBA is determined by the ability of the antioxidants present in the sample to stop the oxidation process, thus inhibiting the formation of the chromogen (**Fig. 33**)

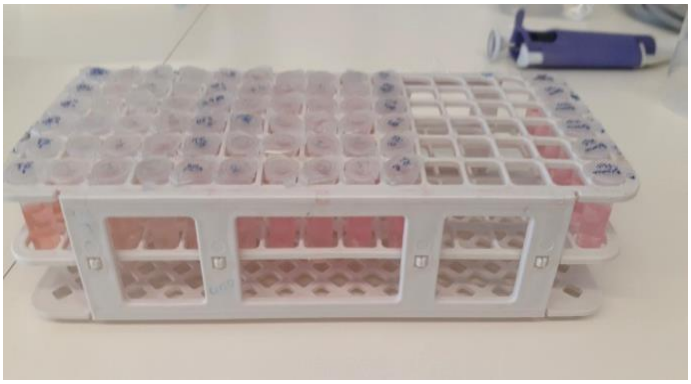

Figure 33: Different levels of MDA-TBA formation (samples on the left are the most antioxidant).

Porcine (*Sus domesticus*) brains were obtained from official slaughtered animals, dissected, and homogenised with a Polytron in ice-cold Tris-HCl buffer (20 mM, pH 7.4), to produce a 1:2 (w/v) brain tissue homogenate that was centrifuged at 3000*g* for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with the extracts (0.2 mL) at different concentrations in the presence of FeSO (10 μM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by TBA (2%, w/v, 0.38 mL), and the mixture was heated at 80 ºC for 20 min.

After centrifugation (3000*g*, 10 min) to remove the precipitated protein, the colour intensity of the MDA-TBA complex in the supernatant was measured by its absorbance at 532nm. The inhibition ratio (%) was calculated as:

$$\textbf{Inhibition ratio (\%) = (A-B) A} \times \textbf{100}$$

Where A and B were the absorbance of the control and the sample solution, respectively.

Oxidative haemolysis inhibition assay (OxHlIA)

To carry out this cellular assay, a blood sample was taken from sheep. The blood sample was centrifuged (Multifuge X1R, Thermo Fisher Scientific; 2900 rpm, 5 min, 10 ºC) and the supernatant was discarded to recover only the erythrocytes.

These were subjected to a first wash with NaCl (150 mM) followed by three washes with phosphate-saline buffer (PBS; pH 7.4), with centrifugation and removal of the supernatant with each wash. Then a 2.8% (v/v) erythrocyte solution in PBS was prepared.

Subsequently, 200 µL of the erythrocyte solution was added to 400 µL of PBS (control), antioxidant extract dissolved in PBS (20 to 0.625 mg / mL) and water (to promote complete haemolysis) using 48-well microplates. After pre-incubating, the microplates at 37 ° C for 10 min with shaking, 200 µL of 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH; 160 mM) was added and the optical density was measured at 690 nm. The microplates were then incubated in the same conditions until complete haemolysis, with measurements every 10 min .[74]

The percentage of the erythrocyte (PE) population that remained intact was calculated as follows:

$$PE\ (\%) = (St - CH0 / S0 - CH0) \times 100$$

Where St and S0 correspond to the optical density of the sample at an instant of time t and 0 min, respectively, and CH0 is the optical density corresponding to complete haemolysis at 0 min. The results were expressed as haemolysis delay time (Δt), which was calculated as follows:

$$\Delta t\ (min) = Ht50\ (sample) - Ht50$$

Ht50 is the time corresponding to 50% of haemolysis (min) obtained graphically from the haemolysis curve for each concentration of antioxidant sample.

Subsequently, correlations were established between the Δt values and the different sample concentrations. From them, the concentration capable of delaying haemolysis in 60 min (EC50 (60 min), mg / mL) and 120 min (EC50 (120 min), mg / mL) was calculated.

Antibacterial activity

In this research, the antibacterial ability of mushroom extracts was investigated by evaluating the zone of inhibition caused by different concentrations of extracts on selected strains of bacteria and fungi.

The bacterial strains were clinical isolates obtained from patients hospitalized in various departments at the Northeastern local health unit (Bragança, Portugal) and Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Five Gram-negative bacteria *Escherichia coli* (isolated from urine), *Proteus mirabilis* (isolated from wound exudate), *Klebsiella pneumoniae* (isolated from urine), *Pseudomonas aeruginosa* (isolated

from expectoration) and *Morganella morganii* (isolated from urine), and three Gram-positive bacteria (*Enterococcus faecalis* (isolated from urine), *Listeria monocytogenes* (isolated from cerebrospinal fluid), and methicillin-resistant *Staphylococcus aureus* (MRSA) (isolated from expectoration) were tested. All these microorganisms were incubated at 37ºC in appropriate fresh medium for 24h before analysis to maintain the exponential growth phase.

Determination of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC):

The MIC determinations on all bacteria were conducted using colorimetric assay according to described by Pires et al, 2018. The samples were first dissolved in 5% (v/v) Dimethyl sulfoxide (DMSO) and 95% of Mueller-Hinton Broth (MHB)/Tryptic Soy Broth (TSB) to give a final concentration of 20 mg/ ml for the stock solution. 190 µl of this concentration was added in the first well (96-well microplate) in duplicate. In the remaining wells place 90 µl of medium MHB or TSB. Then the samples were serially diluted to obtain the concentration ranges (20 at 0.15 mg/mL). To finish 10µl of inoculum (standardized at 1.5×108 Colony Forming Unit (CFU) /ml) was added at all well.

Three negative controls were prepared (one with (MHB)/(TSB), another one with the extract, and the third with medium, antibiotic, and bacteria). One positive control was prepared with MHB/TSB and each inoculum. Ampicillin and Imipenem were used for all Gram-negative bacteria tested and *Listeria monocytogenes*. Ampicillin and vancomycin were selected for *Enterococcus faecalis* and MRSA. The microplates were covered and incubated at 37°C for 24 h. The MIC of samples was detected following addition (40 µl) of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT) and incubation at 37°C for 30 min. MIC was defined as the lowest concentration that inhibits the visible bacterial growth determined by change the coloration from yellow to pink if the microorganisms are viable. For the determination of MBC, 10 µl of liquid from each well that showed no change in colour was placed on solid medium, Blood agar (7% sheep blood) and incubated at 37°C for 24 h. The lowest concentration that yielded no growth determines the MBC. MBC was defined as the lowest concentration required killing bacteria.

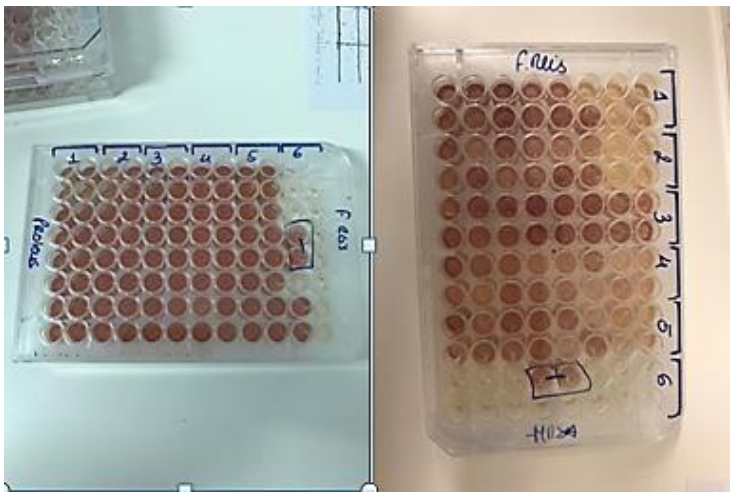

Figure 34: Example of a testing 96 well plate for MIC and MBC determinations

Cytotoxicity

Tumour cell lines

Four human tumour cell lines were used: caco2 (calcium carbonate: immortalized cell line of human colorectal adenocarcinoma cells), AGS (gastric adenocarcinoma) MCF-7 (breast adenocarcinoma) and NCI-H460 (non-small cell lung cancer). Each of the cell lines were placed in a 96-well plate, at an appropriate density (7.5 × 103 cells/well for MCF-7 and NCI-H460, and 1.0 × 104 cells/well for caco2 and AGS) and allowed to attach for 24 h. The cells were then incubated in the presence of different extract concentrations during 48 h.

Afterwards, cold trichloroacetic acid (TCA 10%, 100 µL) was added to bind the adherent cells and further incubated for 60 min at 4 ºC. After the incubation period, the plates were washed with deionised water and dried, and sulforhodamine B solution (SRB 0.1% in 1% acetic acid, 100 µL) was incorporated to each plate well and incubated for 30 min at room temperature. The plates were washed with acetic acid (1%) to remove the unbound SRB and air dried; the bounded SRB was solubilised with Tris (10 mM, 200 µL) and the absorbance was measured at 540 nm (**Fig. 35**) using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA) .[75]

Figure 35: Microplate used in cytotoxicity evaluation

3.10.2. Non-tumour cell line

For the possible hepatotoxicity evaluation, a hepatic cell, designed as PLP2(porcine liver cell line) and Vero (cells are derived from the kidney), are used [76].The liver tissues were washed in Hank's balanced salt solution, containing 100 U/mL of penicillin and 100 mg/mL streptomycin, and then divided into 1×1 mm^3 explants. PLP2 cells were cultured and maintained in DMEM supplemented with 10% foetal bovine serum, 2 mM of non-essential amino acids, 100 U/mL penicillin, and 100 mg/mL streptomycin (Reis et al., 2013). Subsequently were incubated at 37 ºC with a humidified atmosphere (5% CO_2). The medium was changed every two days and the cultivation of cells was continuously monitored every 2-3 days using a phase contrast microscope. Prior to confluence, cells were plated in 96-well plates at an appropriate density (1.0×10^4 cells/well) and treated for 48 h with the different diluted sample solutions.

The same procedure described above for the SRB assay was performed for the growth inhibition. The results were expressed as GI_{50} values (sample concentration that inhibited 50% of the net cell growth), and ellipticine was used as positive control.

Anti-inflammatory activity

Cell line treatment

The anti-inflammatory activity was evaluated in a mouse macrophage-like cell line, designed as RAW 264.7. The assay was performed following a procedure described by ***Taofiq* et al. (2015)**. The mouse macrophage-like cell line RAW 264.7 was cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum, glutamine, and antibiotics at 37 °C under 5% CO_2, in humidified air. Cells were seeded in 96-well plates at 1.5×10^5 cells/well and allowed do attach to the plate overnight. Then, cells were

treated with the different concentrations of each one of the extracts for 1 h. Dexamethasone (50 μM) was used as a positive control for the experiment. The following step was the stimulation with lipopolysaccharides, LPS (1 μg/mL) for 18 h. The effect of all the tested samples in the absence of LPS was also evaluated, in order to observe if they induced changes in nitric oxide (NO) basal levels. In negative controls, no LPS were added. Both extracts and LPS were dissolved in supplemented DMEM.

Nitric oxide determination

For the determination of nitric oxide, Griess Reagent System kit was used, which contains sulphanilamide, N-(1-napthyl) ethylenediamine hydrochloride (NED) and nitrite solutions. A reference curve of the nitrite (sodium nitrite 100 μM to 1.6 μM; y = 0.0066x + 0.1349; R2 = 0.9986) was prepared in a 96-well plate. The cell culture supernatant (100 μL) was transferred to the plate and mixed with sulphanilamide and NED solutions, 5–10 min each, at room temperature. The nitric oxide produced was determined by measuring the absorbance at 540 nm (microplate reader ELX800 Biotek), and by comparison with the standard calibration curve.

Extraction of volatiles compound composition of wild mycorrhizal mushroom:

To analyze the volatile compounds of different fungi, we used two methods, the first method was the extraction of the essential oils by hydro distillation, using a Clevenger apparatus and the second was the Lickens-Nickerson (L.N) method (The difference between this two methods that the Clevenger required a large amount of mushroom for the extraction with addition of distillated water and will be performed by a single stage **(Fig. 36**) , but the L-N required a small amount of mushroom for the extraction with addition of pure water and pentene and will be performed by three stages (**Fig. 37**). The description of all the extraction is in the table 5:

Table 5 : Trials performed with the Extraction of all the mushrooms:

Mushrooms	*B.pinicola1*	*T.terreum*	*L.nuda*	*P.involutus*	*C.odora*	*L.deliciosus*	*T.portentosum*
Extraction with Clevenger	106 g+50 ml of ultra-pure water at 100°C.	100.5 g+50 ml of ultra-pure water at 100°C.	60.02 g+50 ml of ultra-pure water at 100°C.	-	-	-	-
Extraction with L-N	25.5 g +50 ml of distilled water +25 ml of pentene with 30°C of temperature.	28.7 g +50 ml of distilled water +25 ml of pentene with 30°C of temperature	15.2g+50 ml of distilled water +25 ml of pentene with 30°C of temperature	30.8 g+50 ml of distilled water +25 ml of pentene with 30°C of temperature	5.50 g+50 ml of distilled water +25 ml of pentene with 30°C of temperature	11.72 g +50 ml of distilled water +25 ml of pentene with 30°C of temperature	30.8 g+50 ml of distilled water +25 ml of pentene with 30°C of temperature

Note: For those mushrooms (*P.involutus, C.odora , L.deliciosus , T.portentosum*)we did not use the extraction by the Clevenger because we don't have enough mushroom, so we just used L-N .

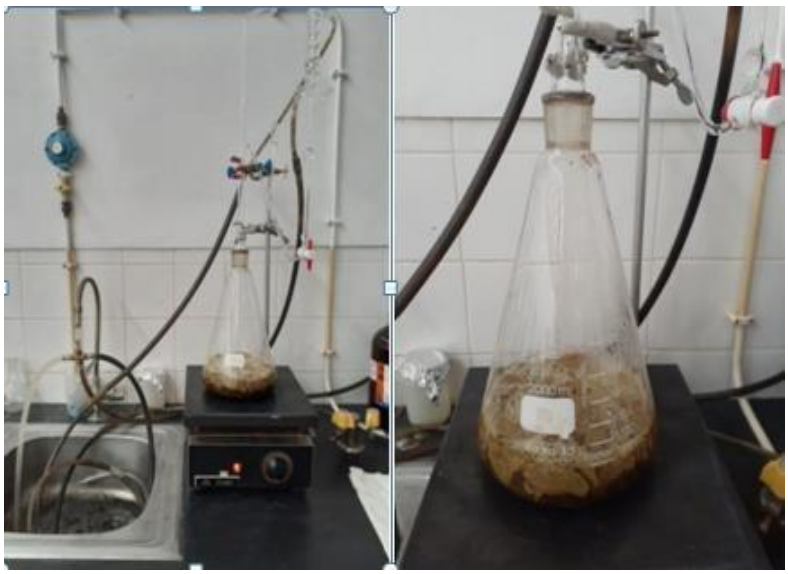

Figure 36: Extraction of volatiles compounds composition by Clevenger.

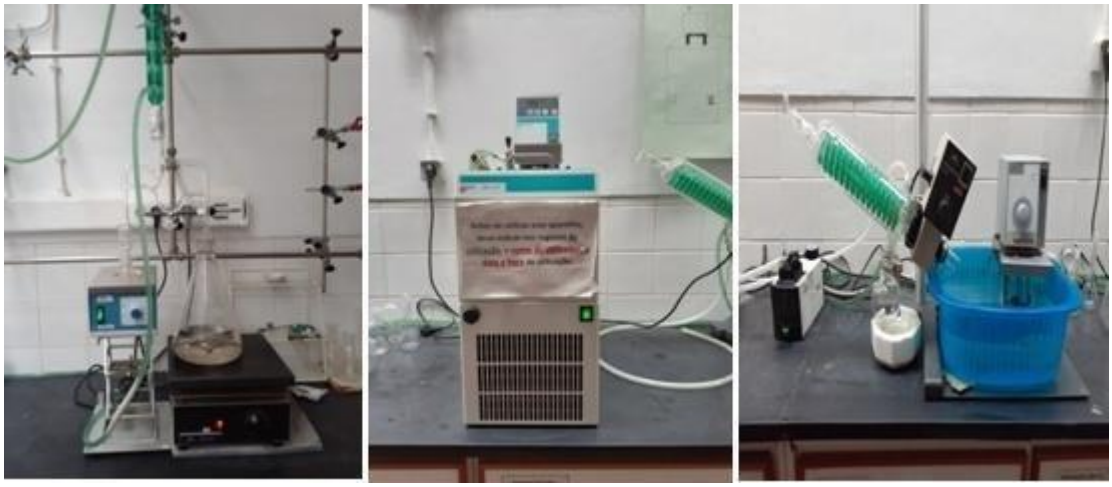

Figure 37: Extraction of volatiles compounds composition by L.N.

Chemical characterization of *Tricholoma terreum*

As abovementioned, the studied mushroom species were chemically characterized regarding their nutritional value, nutrients, and non-nutrients composition, considering that some of the survey compounds are known for their bioactive potential (**Fig. 38**)

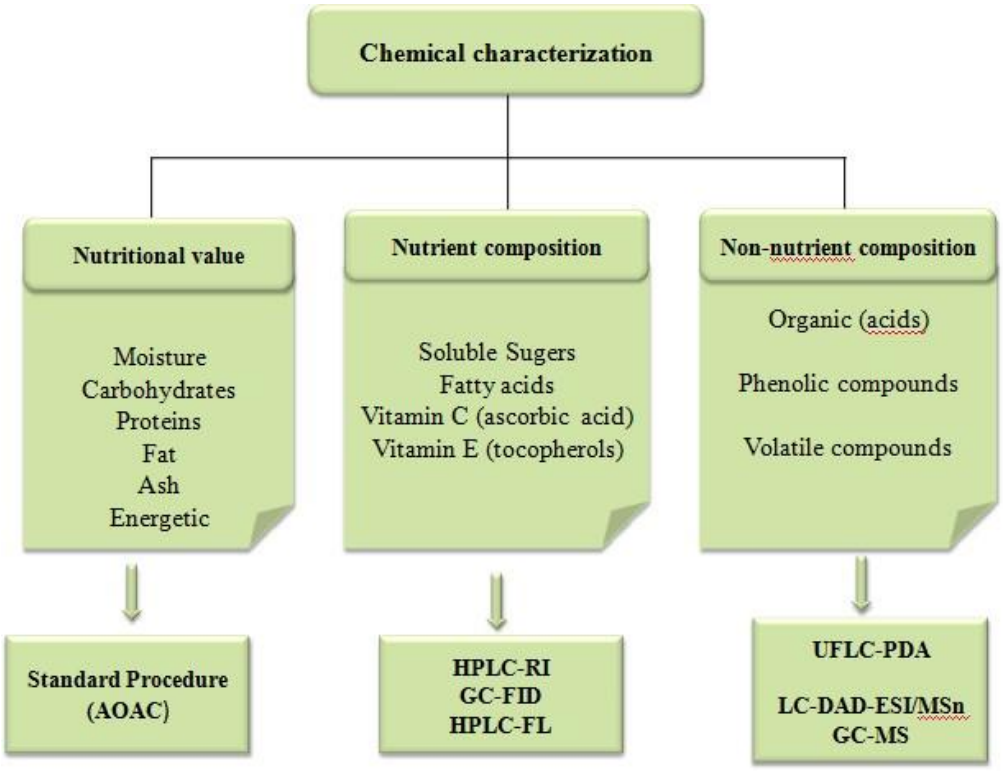

Figure 38: Schematic representation of the evaluated parameters and techniques used to perform the chemical characterization of the mushroom species

Nutritional value

The samples were analyzed for chemical composition (humidity, proteins, fat, carbohydrates, and ash) using the AOAC process (AOAC, 2012). The crude protein content (N *4.38) of the samples was measured using the macro-Kjeldahl method; the crude fat was determined using a Soxhlet apparatus by extracting the known weight of the powdered sample using petroleum ether, the ash content was determined by incineration at 550±15 °C.

1. Ash content determination

For the determination of mineral content, 250 mg of dried sample were added to calcined and previously weighed crucibles. Immediately after, the crucibles were introduced in the muffle (Lenton Thermal designs; ECF12/22), where they were incinerated at 550±15 °C, until a white ash was formed (approximately 20 h). The crucibles were cooled in a desiccator and the weigh was determined.

The results were expressed in ash percent using the following equation:

$$\%ash = \frac{(m_i - m_f)}{m_f} \times 100$$

Where m_i represents the initial mass introduced in the crucible and m_f represent the final mass after the incineration in the muffle (**Fig. 39**).

Figure 39: Muffle used in ash determination

2. Protein content determination by Kjeldahl method

Kjeldahl method allows quantifying the crude protein content based in the nitrogen content of the sample. In this analysis, 250 mg of previously grinded samples were introduced in the digestion tubes with 15 ml of sulfuric acid and two selenium tablets to catalyze the reaction. The tubes were introduced in the digester (FossTM Digestor). After cooling, the digestion tubes were placed in the Kjedahl equipment (**Fig. 40**), where a distillation and a titration were automatically performed.

First, the sample was diluted and neutralized using 25 ml of water and a solution of sodium hydroxide. The second tube is the control (digested white) composed with galleric and sodium hydroxide, the third and fourth are the samples of *T. terreum* with the sodium hydroxide.

Figure 40: Equipment used in the determination of protein content. A - digestor. B - *Kjeldahl* equipment.

3. Determination of soluble sugars

The dried sample powder (1 g) was spiked with melezitose as internal standard (IS, 25 mg/mL) and extracted with 40 ml of 80% aqueous ethanol at 80 °C for 1h30 min, the resulting suspension was centrifuged (at 4800g for 15 min). The combined extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and defatted three times with 10 mL of diethyl ether, successively. After concentration at 40 °C, the solid residues were dissolved in water to a final volume of 5 mL, filtered and transferred 1.5 ml into an injection vial to be analysed by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI).

Figure 41: Equipment (HPLC-RI) used in soluble sugars identification.

4. Fat content determination

To determinate the crude fat the powdered samples (1.5 g) were extracted with petroleum ether using a Soxhlet apparatus (**Fig. 42**). After 7 hours, the extraction was interrupted and the flask, containing the fat solubilized in ether, was removed. Subsequently the solvent was evaporated, and the fat content was determined using the following expression:

$$\%fat = \frac{(m_i - m_f)}{m_f} \times 100$$

Where m_i represents the initial mass and m_f represent the mass of fat obtained in the flask after the evaporation of the solvent.

Figure 42: Soxhlet equipment used in the determination of fat content.

5. Fatty acids profile

Fatty acids obtained after Soxhlet extraction, were subjected to a trans-esterification procedure (methylated with 5 mL of methanol: sulfuric acid 95%: toluene 2:1:1 (v/v/v) for, at least, 12 h in a bath at 50 °C and 160 rpm). Afterwards, 3 mL of distilled water were added in order to obtain phase separation; the fatty acids methyl esters (FAME) were recovered by shaking in a vortex with 3 mL of diethyl ether, and the upper phase was passed through a micro-column of anhydrous sodium sulphate to eliminate water. The sample was recovered in a vial with Teflon and filtered into an HPLC vial and store the samples in the freezer until analysis.

3.14 Statistical analysis

The results obtained throughout the different evaluation studies were analysed by applying statistical tools, selected according to the degree of complexity of the results and considering the defined research purposes. All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 25 (IBM Corporation, New York, USA). Three samples were used for each preparation and all the assays were carried out in triplicate. The results were expressed as mean values ± standard deviation (SD), maintaining the decimal places allowed by the magnitude of the SD. All tests were performed at a 5% significance level. Whenever possible, an analysis of variance (ANOVA) was applied to compare differences among the studied mushroom species or the different co-culture conditions. The typical requirements, homoscedasticity by the Levene test and normal distribution by the Shapiro Wilk's test, were preliminarily performed. The Welch test was applied to verify the existence of statistically significant differences. The ANOVA results were classified using the Tukey HSD test or Games-Howell, when homoscedasticity was verified or not, respectively. When a specific factor was studied using only two levels, a simple student's t-test was used to classify the results.

4. RESULTS AND DISCUSSION

The results obtained were statistically treated and are presented as graphs or tables according to the most suitable form to analyse and visualize them.

Biological material growth

In this work, the *in vitro* culture technique was exploited to produce the species *Pi (P. involutus)* and to obtain enough biomass for co-culture with *C. sativa* roots and subsequently analyze their content in phenolic and volatiles compounds.

After a few days of inoculation on the MMN medium, it was possible to notice the initial growth in most of the Petri dishes. In five weeks, almost the entire surface of the Petri dishes was covered by the mycelium. In other hand, the mycelia that grew in Petri dishes did not cover the entire surface and stopped growing after about a month and a half.

After one month of inoculation and after the co-culture time, it was possible to collect the biomass and weigh the samples to estimate the average growth in each Petri dish and analyse the phenolic compounds for the roots *C. sativa* plants in co-culture with *P. involutus* for different co-culture time (24h and 48h).

Analysis of the phenolic compounds for the roots *C. sativa* plants in co-culturewith *P. involutus* for different time

The phenolic compounds profile of the roots of *C. sativa* alone, and after co-culture with *P. involutus* for 24h and 48 h are shown in the tables 6 and 7:

Table 6 : The phenolic compounds profile of the roots of *C. sativa* **-** Presents the peak characteristics (Rt, retention time, λ_{max} in the visible region, mass spectral data), tentative identifications of phenolic compounds in the ethanolic extracts of the chestnut roots (roots control). Overall, eight different compounds were identified and quantified.

Peak	***Rt (min)***	***λmax (nm)***	***[M-H] (m/z)***	***MS2 (m/z)***	***Tentative identification***
1	4.98	278	783	481(10), 301(41)	Pedunculagin isomer (bis-HHDP-glucose)
2	6.17	278	935	917(87), 633(100), 301(25)	Di-HHDP-galloyl-glucose (casuarictin/potentillin)
3	8.33	245/360	933	915(5), 633 (10), 451(34), 301(13)	Castalagin/vescalagin isomer I
4	12.8	243/350	935	633(12), 301 (22)	Galloyl-bis-HHDP-glucose
5	16.95	246/360	935	633 (19), 301(34)	Galloyl-bis-HHDP-glucose
6	18.49	248/364	937	767(2), 637(8), 467(68), 301(7)	Trigalloyl-HHDP-glucoside
7	21.98	250/364	933	915(5), 633(18), 451(34), 301(6)	Castalagin/vescalagin isomer II
8	26.3	249/366	907	767(5), 607 (35), 467(77), 169(8)	Galloyl-HHDP derivative
9	34.21	251/366	343	328(98), 313(100), 298(66)	Tri-*O*-methylellagic acid

(m/z – Mass to charge ratio)

Calibration curves used: ellagic acid (y = 26719x –317255, R2= 0.999).

In the phenolic profile of the roots of *C. sativa* alone**,** peaks 4 and 5 presented a pseudomolecular ion at *m*/*z* 953 and two MS^2 fragments at *m*/*z* 633 and 301*m/z.* Peaks 3 and

7 also presented a pseudomolecular ion at *m*/*z* 933 and MS^2 fragments at *m*/*z* 915, 633, 451 and at 301*m/z,* but with different isomer (isomer I and II).

For the other peaks present different phenolic compounds: for the peak 1 releasing two MS^2 fragment at m/z 783, corresponding to Pedunculagin isomer (bis-HHDP-glucose, peak 2 ($[M-H]^-$ at *m/z* 953) were assigned as Di-HHDP-galloyl-glucose (casuarictin/potentillin), peak 6($[M-H]^-$ at *m/z* 973) were assigned as Trigalloyl-HHDP-glucoside, peak 8 at *m*/*z* 907 releasing four MS^2 fragment at m/z 767, 607, 467, 169, corresponding to Galloyl-HHDP derivative and finally peak 9 presented a benzophenones at (*m/z* 343) being assigned as Tri-*O*-methylellagic acid, taking into account the previously reported fragmentation pattern reported by Berardini, Carle, & Schieber (2004) in peels of *Mangifera indica*. Thus, to the best of our knowledge those compounds were not previously reported in the chestnut roots.

Table 7 : Phenolic compounds profile of the roots co-cultured with *P. involutus* for 24h and 48h - Presents the peak characteristics (Rt - retention time, λ_{max} in the visible region, mass spectral data), tentative identifications of phenolic compounds in the ethanolic extracts of the roots of the chestnut (roots). (Roots co-culture with *P. involutus* for 24h and 48h).

Peak	***Rt (min)***	***λmax (nm)***	***[M-H] (m/z)***	***MS2 (m/z)***	***Tentative identification***
1	4.98	278	783	481(10), 301(41)	Pedunculagin isomer (bis-HHDP-glucose)
2	6.17	278	935	917(87), 633(100), 301(25)	Di-HHDP-galloyl-glucose (casuarictin/potentillin)
3	8.33	245/360	933	915(5), 633 (10), 451(34), 301(13)	Castalagin/vescalagin isomer I
4	12.8	243/350	935	633(12), 301 (22)	Galloyl-bis-HHDP-glucose
5	16.95	246/360	935	633 (19), 301(34)	Galloyl-bis-HHDP-glucose
6	18.49	248/364	937	767(2), 637(8), 467(68), 301(7)	Trigalloyl-HHDP-glucoside
7	21.98	250/364	933	915(5), 633(18), 451(34), 301(6)	Castalagin/vescalagin isomer II
8	26.3	249/366	907	767(5), 607 (35), 467(77), 169(8)	Galloyl-HHDP derivative
9	34.21	251/366	343	328(98), 313(100), 298(66)	Tri-*O*-methylellagic acid

Calibration curves used: ellagic acid (y = 26719x –317255, R2= 0.999).

Analyzing the results of the three tables, the same phenolic compounds and the same results found in the roots control and the roots with symbiosis (24h and 48h), so we can still conclude that, when in symbiosis for short time contact (24h and 48h), the catching effect of the roots of *C. sativa* is the same compared to the effect observed when growing alone.

According to the literature, some authors found different profiles in the studied species, and were able to identify different phenolic compounds, such as vescalagin (0.06 to 0.10 mg/g FW), castalagin (0.41 to 0.82 mg/g FW), tannin T1 (0.06 to 0.09 mg/g FW), tannin T2 (0.05 to 0.09 mg/g FW), acutissimin A (0.05 to 0.08 mg/g FW) acutissimin B (0.41 to 0.51 mg/g FW) and ellagic acid derivatives (0.11 to 0.16 mg/g FW) in the outer shell of chestnuts (Vasconcelos et al.. 2009)[76]. Some authors have identified some phenolic compounds on chestnut fruits, namely, syringic+caffeic acids (0.002 to 0.02 mg/g FW), vanillic acid (0.15 to 0.92 mg/g FW), rutin (0.005 to 0.026 mg/g FW), catechin (0.024 to 0.13 mg/g FW), chlorogenic acid (0.004 to 0.12 mg/g Fw), p-coumaric acid (0.004 to 0.033 mg/g FW), ferulic acid (0.004 to 0.015 mg/g FW) and naringin (0.007 to 0.021 mg/g FW. Longer time of co-culture should be needed to induce changes in the response of the rooting system to the symbiosis by phenolic acids production[77].

4.1.2 The phenolic compounds of *Paxillus involutus*

The phenolic compounds profile of the fungus alone, and after co-culture with the roots of *C. sativa* for 24h and 48 h are shown in the table 8. The same results were obtained for the three samples, meaning that no effect of the symbiosis induction for 24h and 48h was found on the profile of phenolic acids, as already noticed for the roots of *C. sativa.*

Table 8 : Phenolic compounds profile of *P. involutus*: *Pi* control, *Pi* co-culture 24h and 48h - Presents the peak characteristics (retention time, λ_{max} in the visible region, mass spectral data), tentative identifications of phenolic compounds in the ethanolic extracts of the fungi (*Pi* control, *Pi* co-culture 24h and 48h): The same results were obtained for the three samples:

Peak	***Rt (min)***	***λmax (nm)***	***[M-H]⁻ (m/z)***	***MS2 (m/z)***	***Tentative identification***
3	7.74	280	313	-	Degradation product of protocetraric acid or physodalic acid
4	9.3	279	297	-	Degradation product of unkwon compound
6	11.03	362	311	-	Degradation product of protocetraric acid or physodalic acid
8	14.54	280	267	-	Degradation product of protocetraric acid or physodalic acid after 313 and 311
9	17.2	243/320	251	-	Degradation product of protocetraric acid or physodalic acid after 313, 311, and 267

NOTE: we have no calibration curves to identify those compounds

Regarding the fungus *P. involutus*, the identification was very difficult, because we don't have any calibration curve, so we have recourse to articles, to make the comparison and know at least one of these phenolic compounds.

We found fragments of compounds that should be present, namely acids (phenylethanol); it is a fragment of a real compound that we cannot identify and quantify this compound, as we have no calibration curves. We made the identification based on the following article: "Secondary metabolites of the lichen *Hypogymnia physodes* (L.) Nyl and their presence in spruce (*Picea abies* (L.) H. Karst) bark".

In this article, we found a fragment of protocetraric acid or physodalic acid ($[M-H]^-$ after (m/z 313, 311 and 267), without the identification of the real compound.

The results presented previously by some authors [78], shows that an ion fragments for the compound hp3 were observed at m/z 311 and 267, respectively. The loss of all the

above-mentioned groups in an alternative sequence resulted in the following fragments in the mass spectrum of compound hp1: m/z 399, 355, 339, 311, 295 and 251. It is worth noting that both compounds hp1 and hp3 have a partially coincident fragmentation pattern.

We can conclude that the initial responses of the plant and fungus to the contact with the symbiont doesn´t induce changes in the phenolic acid profiles of the partners. Longer periods of co-culture must be tried to evaluate the potential role of these compounds during the recognition process of plant and fungus.

Analysis of natural bioactive compounds of wild mycorrhizal mushroom:

Phenolic compounds

The phenolic compounds profile of the wild mushrooms collected (*T.equestre, T.terreum, B.pinicola 2, B.pinicola 3, L.deliciosus)* are shown in table 9. From this table we notice that all the 4 mushroom samples only presented 1 or 2 phenolic compounds.

Table 9 : Presents the peak characteristics (retention time, λ_{max} in the visible region, mass spectral data), tentative identifications of phenolic compounds in the ethanolic extracts of all the mushrooms (*TE, TT, BP2, BP3, LD*).

Peak	***Rt (min)***	***λmax (nm)***	***[M-H]⁻ (m/z)***	***MS2 (m/z)***	***Tentative identification***
1	5.1	258	153	109(100)	Protocatechuic acid
2	8.06	272	137	93(100)	*p*-hydroxybenzoic acid

Calibration curves used: procatechuic acid (y=214168 xs+27102, R2= 0.999) and *p*-hydroxybenzoic acid (y=208604 xs+173056, R2= 0.999)

Peak 1 presents a pseudomolecular ion at *m/z* 153 corresponding to Protocatechuic acid and one MS^2 fragments at109 *m/z.* Peak 2 also presents a pseudomolecular ion corresponding to *p*-hydroxybenzoic acid at *m/z* 137 and MS^2 fragments at 93 *m/z.*

These results are in agreement with the ones reported in previous studies on Portuguese samples of *B. edulis, L. deliciosus and T. terreum*[79], Protocatechuic, p-

hydroxybenzoic and cinnamic acids were found in all the three samples, while p-coumaric acid was only found in *B. edulis* contributing to the highest amount in total phenolic compounds presented by this latter, as well as the highest content in p-hydroxybenzoic acid.

According to previous literature the total phenolic content in methanol and the aqueous extracts of *T. equestre* was calculated using the calibration curve of gallic acid (0.999). The total content of phenols in the methanol extract was 14.07 ± 0.38 µg GA/mg of extract, and the total content of phenols in the aqueous extract was 12.07 ± 0.18 µg GA/mg of extract. Thus, we concluded that the methanol extracts of *T. equestre* were richer in phenols than the aqueous extracts.[79]

Genetic differences between organisms, different natural environments, diverse environmental conditions during mushroom growth and maturation, degree of damage, or air pollution can all contribute to differences in the qualitative and quantitative composition of phenolic compounds [82].

As a result, lyophilization was suggested as a drying process because it had the least impact on the levels of these compounds. It can be concluded that edible mushrooms can be a valuable dietary source of antioxidants based on the obtained results confirming the existence of phenolic compounds.[83]

4.2.2. Organic acid

Some of the organic acids found primarily in mushrooms have biological activity in addition to their functions in primary metabolism. The antioxidant properties of citric and quinic acids are well known [84]. Fumaric acid has been described as an effective treatment for psoriasis and inflammation, as well as a potential neuro- and chemoprotector [85]. Malic acid, which is used in food additives and as an excipient in the pharmaceutical and polymer industries, has bactericidal properties .[86]

The amounts of organic acids present in the studied edible mushrooms are presented in Table 10:

Table 10 : Organic acids composition of the studied mushroom species (mean ± SD)

Organic acid (g/100g dw)	*B. pinicola (2)*	*B. pinicola (3)*	*L. deliciosus*	*T. equestre*	*T. terreum*
Oxalic acid	0.59 ± 0.02^{d}	3.38 ± 0.01^{a}	1.9 ± 0.2^{b}	0.75 ± 0.02^{c}	1.9 ± 0.1^{b}
Quinic acid	nd	nd	nd	2.77 ± 0.03^{b}	4.5 ± 0.3^{a}
Malic acid	0.260 ± 0.005^{e}	0.67 ± 0.04^{d}	3.1 ± 0.3^{b}	2.40 ± 0.01^{c}	3.19 ± 0.02^{a}
Citric acid	nd	nd	nd	0.82 ± 0.04^{a}	0.173 ± 0.005^{b}
Fumaric acid	tr	tr	tr	tr	tr
Total organic acids (kcal/100gdw)	$\mathbf{0.85 \pm 0.02^{e}}$	$\mathbf{4.04 \pm 0.02^{d}}$	$\mathbf{4.9 \pm 0.1^{c}}$	$\mathbf{6.75 \pm 0.01^{b}}$	$\mathbf{9.7 \pm 0.4^{a}}$

Nd: not detected; tr- trace. In each row, different letters mean statistically significant differences among the samples. Significance level was set as $p<0.05$. Calibration curves used: oxalic acid (y=1E+07x+231891, , R2= 0.999), Quinic acid (y=671557x+14583, , R2= 0.9998),Malic acid (y=950041x+6255.6, , R2= 0.999),citric acid (y=1E+06x-10277, , R2= 0.9997),Fumaric acid (y=1E+0.8x+614399, , R2= 0.9986).

Five organic acids were identified, specifically fumaric (only in trace amounts) acids. There was not a main organic acid for all studied mushrooms. In general terms, malic acid was found in higher amounts in *T. terreum* (3.19 g/100g dw) and *L. deliciosus* (3.1 g/100g dw), citric acid in *T. equestre* (0.82 g/100g dw), oxalic acid in *B. pinicola* 3 (3.38 g/100g dw), and quinic acid in *T. terreum* (4.5 g/100g dw). It should be noted that the chemistry of an organism may also vary according to the conditions under which it develops [87].

This difference between all the samples may be due to the environmental conditions. Moreover, once the organic acids are constituents of the Krebs cycle, and being wild mushrooms more prone to adverse conditions, these may have higher energy requirements comparing to mushrooms growing under controlled conditions.

Some authors identified that although morphologically similar, fungi metabolites may be very different. Some metabolites may be produced by all the varieties of a particular species, while others may be specific metabolites of an organism [88].

Mushroom growth

Comparison of the growth of different wild mushroom in different culture media (PDA and MMN):

All mushroom samples were analysed in different culture media (MMN and PDA), to get knowledge on the growth preference medium of each one:

The growth of *P. involutus* in different culture media

The average radius of growth of *P. involutus* was calculated for each week of culture and is shown in Table 11 and Fig.43.

Table 11 : Average mycelium growth in diameter in different culture medium for *P. involutus:*

				MMN				***PDA***		
Time (week)	1	2	3	4	ΔG	1	2	3	4	ΔG
Average radius (cm)	0.859	1.407	1.591	1.971	1.112	0.685	0.685	0.685	0.685	*0*

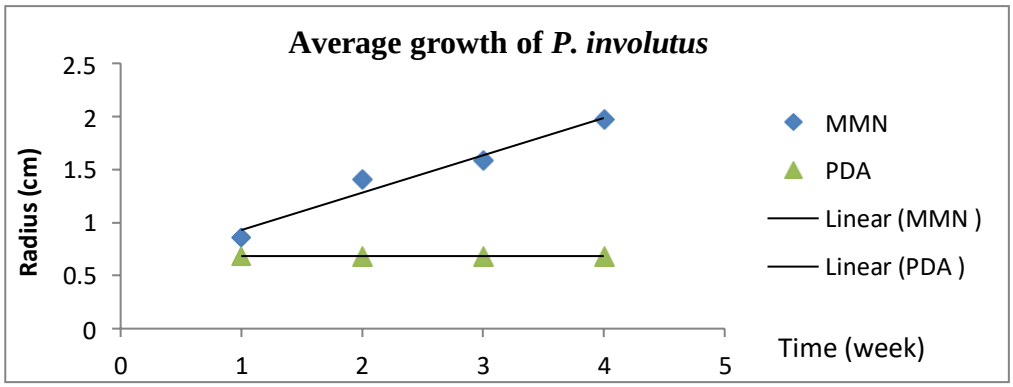

Figure 43: Average mycelium growth of *P. involutus* along 4 weeks

Through the analysis of the obtained results, it can be verified that the radial growth of *P. involutus* was different depending on the medium (MMN and PDA). The growth was higher in MMN medium (the average growth was 1,112 cm in radius in 4 weeks in MMN medium) and it didn't grow at all in PDA from the first week on (the average is constant

from the first week 0, 6853 until the fourth week). With these results, it is possible to conclude that the MMN medium is more conducive for the growth of *P. involutus*.

The growth of *P. involutus* (Brg) in different culture medium

The average radius of growth of the isolated *P. involutus (Brg strain)* was calculated for each week of culture and is shown in Table 12 and Fig.44.

Table 12 : Average mycelium growth in different culture medium for *P. involutus* (Brg):

	MMN					***PDA***				
Time (week)	1	2	3	4	ΔG	1	2	3	4	ΔG
Average radius (cm)	1.192	2.234	3.009	3.636	*2.444*	0.936	0.936	0.936	0.936	*0*

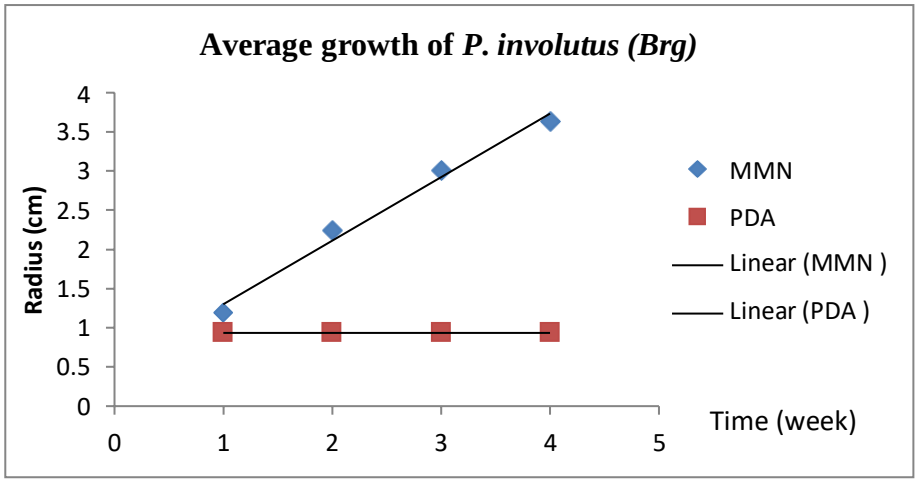

Figure 44: Average mycelium growth of *P. involutus (Brg)* along 4 weeks

Through the analysis of the obtained results, it can be verified that the production of *P. involutus* (Brg strain) developed in different ways, depending on the medium (MMN and PDA), the growth was higher in MMN medium (the average was increased by 2,444 cm in 4 weeks, it is triple in comparison with the first week) compared to PDA medium (the average is constant from the first week 0, 9357 until the fourth week). These findings suggest that the MMN medium is better for *P. involutus* growth (Brg).

The growth of *L. nuda* in different culture medium

In Table 13 and Fig. 45 are shown the average radius of growth of the isolated *L. nuda that* was calculated for each week of culture.

Table 13 : Average mycelium production in different culture medium for *L. nuda*

	MMN					***PDA***				
Time (week)	1	2	3	4	*ΔG*	1	2	3	4	ΔG
Average radius (cm)	1.043	2.146	2.267	2.382	*1.339*	0.707	1.112	1.180	1.350	*0.643*

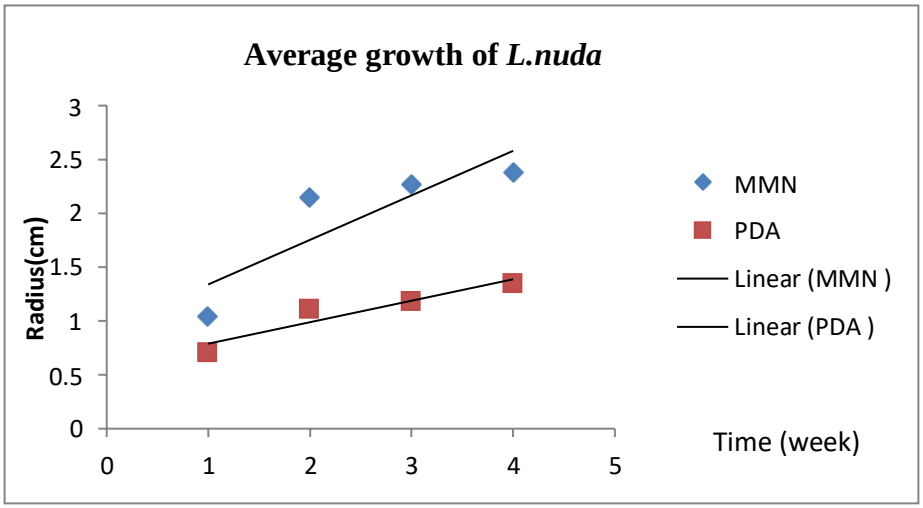

Figure 45: Average mycelium growth of *L. nuda* along 4 weeks

It is clear from the study of the results that the production of *L. nuda* evolved in different ways depending on the medium (MMN and PDA), the growth was higher in MMN medium (the average growth was 1,339 cm in radius in 4 weeks) compared to PDA medium (the average growth was 0.643 cm in radius in 4 weeks). With these results, it is possible to conclude that the MMN is a more conductive media then PDA for the growth of *L. nuda*.

The growth of *C. odora* in different culture medium:

In Table 14 and Fig. 46 are shown the average radius of growth of the isolated *C.odora that* was calculated for each week of culture.

Table 14 : Average mycelium production in different culture medium for *C. odora:*

				MMN					***PDA***	
Time (week)	1	2	3	4	ΔG	1	2	3	4	ΔG
AVERAGE	2.559	4.114	4.145	4.145	1.586	1.109	2.825	3.116	3.422	2.313

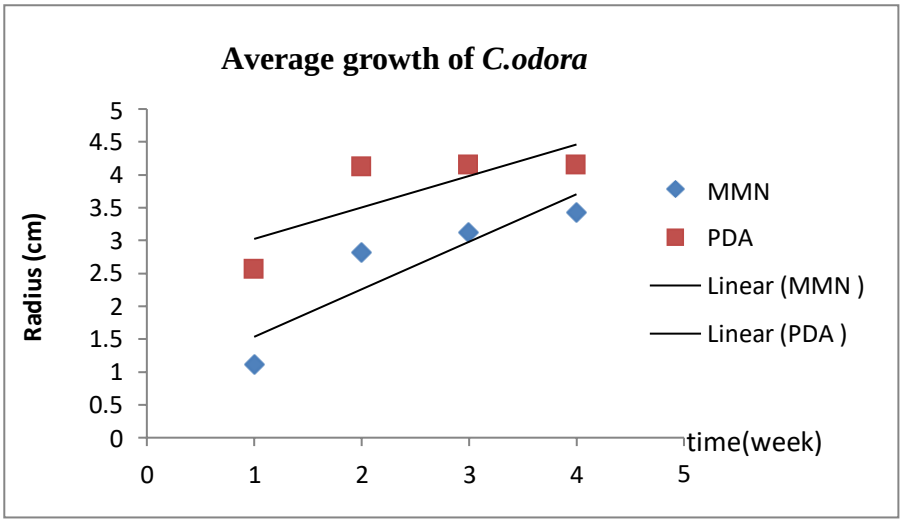

Figure 46: Average mycelium growth of *C. odora* along 4 weeks

It is clear from the examination of the findings that the growth rate in PDA medium was higher (the average growth was 2.313 cm in radius in 4 weeks, it is double in comparison with the first week) compared to MMN medium (the average growth was 1.586 cm in radius in 4 weeks). With these findings, it's conceivable to conclude that the PDA medium is better for *C. odora* growth.

The growth of *L. deliciosus* in different culture medium:

In Table 15 and Fig. 47 are shown the average radius of growth of the isolated *C.odora that* was calculated for each week of culture.

Table 15 : Average mycelium production in different culture medium for *L. deliciosus*:

				MMN				***PDA***		
Time (week)	1	2	3	4	ΔG	1	2	3	4	ΔG
AVERAGE	0.876	1.466	1.466	1.466	0.606	0.835	0.835	0.967	0.967	0.1 32

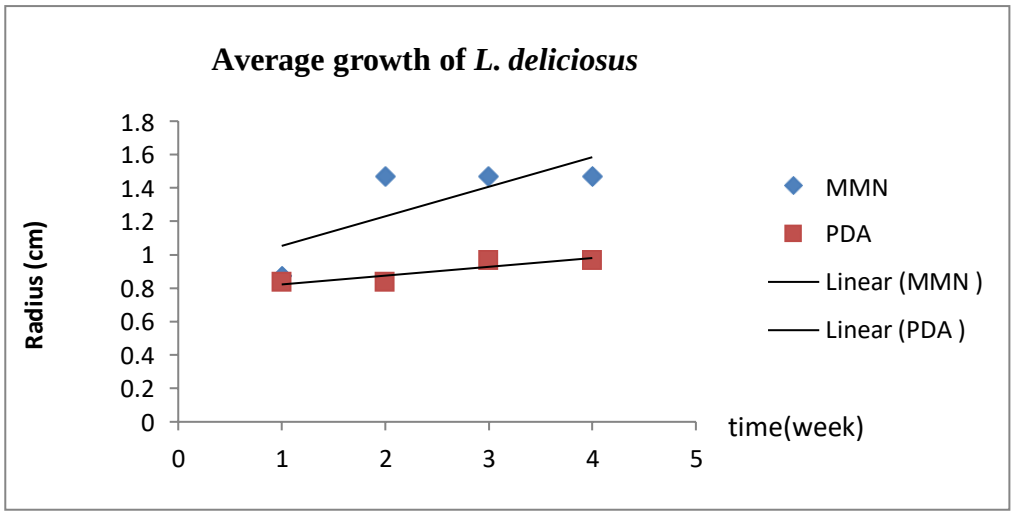

Figure 47: Average mycelium growth of *L.deliciosus* along 4 weeks

The production *L. deliciosus* developed in different ways, depending on the medium (MMN and PDA), in the MMN medium, the rate of growth was faster (the average growth was 0.606 cm in radius in 4 weeks,) compared to PDA medium (the average growth was 0.132 cm in radius in 4 weeks). With these results, it is possible to conclude that the MMN medium is more conducive to growth of *L. deliciosus*.

Bioactivity of edible mushroom

Antioxidant activity

The results obtained for antioxidant activity of the edible mushrooms (*B. pinicola2*; *B. pinicola 3, L. deliciosus , T. equestre , T. terreum*), collected and studied are presented in Table 16 and 17:

TBARS assay

Table 16 : Antioxidant activity (EC_{50} values, µg/mL) of the samples.

	TBARS ASSAY
B.pinicola 2	401 ± 17^{c}
B.pinicola 3	270 ± 10^{d}
L.deliciosus	362 ± 23^{c}
T.equestre	1582 ± 115^{a}
T.terreum	742 ± 53^{b}
Trolox (standard)	3.73 ± 1.90

In the corresponding column, different letters mean statistically significant differences among the samples. Significance level was set as $p<0.05$.

Oxidative haemolysis inhibition assay (OXHLIA)

The Δt values (min) resulting from the half haemolysis time (Ht_{50} values) obtained from the haemolytic curves of each extract sample concentration minus, the Ht_{50} value of the PBS control were correlated to the respective extract concentration to obtained IC_{50} values (µg/mL) (Silva de Sá et al., 2019), which were calculated for a time period of 60 and 120 min, *i.e.*, extract concentration required to protect 50% of the erythrocyte population from the haemolytic action of AAPH for 60 and 120 min.

Table 17 : Antioxidant activity (IC_{50} values, µg/mL) of the samples.

	OxHLIA		***p-Value***
	Δ*t* 60 min	**Δ*t* 120 min**	
L.deliciosus	na	na	-
T.equestre	na	na	-
T.terreum	na	na	-
B.pinicola 2	146±2	854±28	<0.001
B.pinicola 3	104±3	659±19	<0.001
Trolox (standard)	19.7±0.3	41.8±0.4	-

The inhibition concentration at 50 % inhibition (IC50) was the parameter used to compare the radical scavenging activity. A lower IC50 meant better radical scavenging activity. There was statistically significant difference between extracts and control ($P < 0.05$).

The results for the antioxidant potential of the studied mushrooms are presented in Table 16 and 17. All the studied samples revealed antioxidant potential for the TBARS assay but not for the oxhilia. There are different pathways for the molecules exert their antioxidant potential (e.g.: scavenging activity/electron donner, inhibit the lipid peroxidation), so it seems that the ones that give results only for TBARS may exert their activity by inhibiting the cell lipid peroxidation.

The results obtained for TBARS formation inhibition corroborate the results obtained by the OxHlIA, since the EC_{50} (270 µg/mL) and the IC 50(104 for 60 min and 659 for 120 min) values obtained for *B. pinicola 3* is strikingly lower, However, it has been concluded that Bp3 have the high potential antioxidant activity than the other mushrooms.

These results are in agreement with the ones reported by (Croft, 1999), *B. pinicola* 3 have the highest levels of phenolics (388 mg GAE/g extract). (Phenolic compounds have antioxidant properties and can act as free radical scavengers, hydrogen donators and singlet oxygen quenchers.

These results are a strong indicator of the potential application of the ethanolic extracts of *B. pinicola* in pharmaceutical related uses. A similar level of antioxidant activity was previously found in Turkish samples of the species studied herein .[89]

This is not the same case for *T. equestre* which presents the high rate of EC50, therefore the lowest antioxidant activity.

According to the previous literature in 2009, *Boletus edulis* shown the highest phenolic compounds in acetone extract at a 8.14 μg of pyrocatechol equivalent, followed by acetone extract of *Boletus aestivalis* with 6.73 μg of pyrocatechol equivalent,also a good flavonoid content was found in the acetone extract of *Boletus edulis* (4.93 μg of rutin equivalent),The IC50 values was correlated with total phenolic. Notably negative correlation was established between the phenols and IC50 values of antioxidant activities (r = -0.93). There is a good negative correlation between flavonoid compounds of the tested extracts and IC50 values of antioxidant activities (r = -0.83). These negative linear correlations prove that the sample with highest antioxidant contents show higher antioxidant activity with lowest IC50 values.[90]

There are some reports on the antioxidant potential of *T. equestre* have already shown that the occurrence of phenolic compounds in *T. equestre* has been related to its antioxidant capacity. However, this behavior appears to be minimal, which may mean that the content of antioxidant-active compounds is low or that there are substances that intensify oxidation processes in addition to antioxidant-active compounds. [91]

At the same time, some authors describing this specie as a non-edible fungus, Compared to other edible fungi species, such as *Agaricus terreus* (*T. terreum*) (IC50 = 3000 g/mL) and *Hypsizigus marmoreus* (ethanolic extract, IC50 = 4190 g/mL; hot water extract, IC50 = 6480 g/mL). [92]

Antimicrobial activity

In what concerns the antibacterial activity (**Table 18**), none of the assayed samples showed bactericidal activity up to the maximum assayed concentration (20 mg/mL). Nonetheless, the same extracts inhibit the bacterial growth, especially among Gram-positive bacteria. Within this group, MRSA showed the highest sensitivity, while *Listeria monocytogenes* turned out to be the most resistant. In turn, *Escherichia coli* were the most sensitive species among Gram-negative bacteria, while *Proteus mirabilis* and *Pseudomonas*

aeruginosa stood out as the most resistant. Considering the assayed samples *B.pinicola 3* showed the highest antimicrobial activity, which is in agreement with its higher content in phenolic compounds. However, this correlation was not observed for all assayed mushrooms (MIC values for *B.pinicola 2* were closer to the ones from *T.terreum, L.deliciosus and T.equestre* than those from *B.pinicola 3*), which indicates that other compounds besides phenolics contributed to the antibacterial activity.

Table 18 : Antimicrobial activity of the ethanolic extracts of different mushrooms

	BP3		BP2		TT		LD		TE		Ampicillin (20mg/mL)		Imipenem (1mg/mL)		Vancomycin (1mg/mL)	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria																
Escherichia coli	**2.5**	>20	**2.5**	>20	**10**	>20	**5**	>20	**10**	>20	<0.15	<0.15	<0.078	<0.078	n.t.	n.t.
Klebsiella pneumoniae	**20**	>20	>20	>20	>20	>20	>20	>20	>20	>20	10	20	<0.078	<0.078	n.t.	n.t.
Morganella morganii	**20**	>20	**20**	>20	>20	>20	**20**	>20	**20**	>20	20	>20	<0.078	<0.078	n.t.	n.t.
Proteus mirabilis	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	<015	<0.15	<0.078	<0.078	n.t.	n.t.
Pseudomonas aeruginosa	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	0.5	1	n.t.	n.t.
Gram-positive bacteria																
Enterococcus faecalis	**20**	>20	**20**	>20	>20	>20	**20**	>20	**10**	>20	<0.15	<0.15	n.t.	n.t.	<0.078	<0.078
Listeria monocytogenes	**20**	>20	**10**	>20	>20	>20	**20**	>20	**20**	>20	<0.15	<0.15	<0.078	<0.078	n.t.	n.t.
MRSA	**5**	>20	**10**	>20	**20**	>20	**20**	>20	**20**	>20	<0.15	<0.15	n.t.	n.t.	0.25	0.5

MIC: The minimum inhibition concentration (MIC) for bacteria and fungi
MBC: The minimum bactericidal concentration

According to some studies,that already shown that the antimicrobial activity of the *T. equestre* cyclohexane extract and the *T. equestre* dichloromethane extract with respect to *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (29212), *Escherichia coli* (ATCC 10536), and *Klebsiella pneumoniae* (NCIMB 9111) is still stronger than that of most of the previously examined extracts.[93]

Ancient research, has shown that the antimicrobial effect of extract of *L. deliciosus* was tested against four species bacteria (*S. aureus, E. coli, P. aeruginosa* and *K. pneumoniae*) and one species fungus (*C. albicans*). The inhibition zones of *L. deliciosus* which were obtained against all test bacteria were in the range of 9-30 mm. The highest inhibitory activity was determined against *P. aeruginosa* (30±0.0 mm inhibition zone diameter) with acetone and methanol extraction of *L. deliciosus*. On the other hand, the weakest inhibitory activity was determined against *S. aureus* and *C. albicans* (9±0.0 mm inhibition zone diameter) with DMSO and distilled water extraction of mushroom.[95]

Cytotoxicity activity

The potential cytotoxicity (**Table 19**) of the ethanolic extracts of different mushrooms was evaluated using four human tumor cell lines and a non-tumor cell line isolated from porcine liver.

Table 19 : Cytotoxicity of the mushroom extracts in non-tumor and tumor cells.

	GI_{50} (µg/mL)					
	PLP2	Vero	MCF-7	NCI-H460	Caco-2	AGS
BP2	> 400	> 400	> 400	> 400	368 ± 16[a]	> 400
BP3	> 400	> 400	> 400	> 400	315 ± 12[b]	> 400
LD	> 400	> 400	> 400	> 400	> 400	> 400
TE	> 400	> 400	> 400	> 400	> 400	> 400
TT	> 400	> 400	70.9 ± 0.5	59 ± 2	53.1 ± 0.3[c]	> 400
Ellipticine	1.4 ± 0.1	1.41 ± 0.06	1.02 ± 0.02	1.01 ± 0.01	1.21 ± 0.02	1.23 ± 0.03

In the corresponding column, different letters mean statistically significant differences among the samples. Significance level was set as $p<0.05$.

The results for the cytotoxic activity of the studied mushrooms are presented in Table 19.For *L.deliciosus* and *T.equestre* the cytotoxicity was not determined because the GI50 value was higher than 400 (µg/mL) (those essays will be repeat it as a futur work with higher concentration of our extract).

For *B.pinicola* 2 and 3, the cytotoxicty was not determined for the tumor cell lines (PLP 2 and Vero), because (400 (µg/mL) was the highest concentration tested, the two samples present a cytotoxic activity just for the tumor cell line (caco2) but with the lower effect because the value of GI50 was very higher (368 ± 16^{a} for *B.pinicola* 2 and 315 ± 12^{b} for *B.pinicola* 3).

For *T.terreum* the cytotoxicity was not determined for the non -tumor cell lines, because the GI50 value was higher than 400 (µg/mL), but it have a cytotoxic effect against tumor cell lines (MCF-7, NCI-H460, caco2), especially caco2 because it present the lower value of GI50 (53.1 ± 0.3^{c}).

Anti-inflammatory activity

The anti-inflammatory activity was evaluated in a RAW 264.7 cell line.

The results of the anti-inflammatory activity are not shown since all extracts had an IC_{50} greater than 400µg/mL (> 400). (The IC50 value responsible for 50% inhibition of NO production estimated).

Moro et al. analysed six mushroom species from Spain (*Boletus edulis, Cantharellus cibarius* Fr., *Craterellus cornucopioides* (L.) Pers., *Lactarius deliciosus*, (L. ex Fr.) S.F. Gray *Agaricus bisporus* and *Pleurotus ostreatus*) in what concerns the anti-inflammatory activity of their methanolic extracts through NO production in LPS stimulated RAW 264.7 cells. At a concentration of 0.5 mg/mL, *Agaricus bisporus*, and *Lactarius deliciosus* showed 35%, and 40% of NO production inhibition, respectively. The release of TNF-α production was estimated by ELISA kits, but methanolic extracts showed no reduction of TNF-α production in the macrophages.[96]

Different research, investigated the anti-inflammatory function of ethanolic extracts of ten wild and four cultivated mushroom species from Portugal's northeast. The amount of NO generated by RAW 264.7 macrophage cells was measured using the griess reagent assay after they were stimulated with LPS. In opposition of the result of Moro et al., ethanolic extracts of *Agaricus bisporus* and *Boletus edulis* did not display activity.[63]

Nutritional value of *Tricholoma terreum*

The results for the Nutritional value composition of the studied edible mushroom (*T.terreum*) are presented in Table 20 to 22:

Table 20 : Nutritional value of *Tricholoma terreum* (Schaeff.) P.Kumm. (mean ± SD)

	Tricholoma terreum
Moisture (g/100 g dw)	92.2 ± 0.6
Total fat (g/100 g dw)	6.2 ± 0.5
Protein (g/100 g dw)	16.86 ± 0.08
Ash (g/100 g dw)	0.0160 ± 0.0004
Carbohydrates (g/100 g dw)	77.0 ± 0.4
Energy value (kcal/100 g dw)	431 ± 2

Carbohydrates were the main macronutrient present in the specie with highest level (77.0 ± 0.4), which agrees with literature that states that carbohydrates constitute about one-half of mushroom dry matter [97]. Carbohydrate's content includes also dietary fiber, such as the structural polysaccharides β glucans, chitin, hemicelluloses and pectin substances. Since *T. terreum* is a thick, hard and woody mushroom, this could explain the particularly high carbohydrates content.

Mushrooms are said to be a decent source of protein, with an amino acid profile similar to that of animal protein. Indeed, the most abundant amino acids in mushrooms have been identified as leucine, valine, glutamine, glutamic, and aspartic acids. The crude protein content calculated for the studied specie varies between 16.94 and 16.78 g/100 g dw[98].

The remaining sample results are within a narrow range of values published in the literature (22.8 and 24.9 g/100g dw). The estimated medium value of the content of crude protein of mushrooms is approximately 20 % of total dry matter .[97]

Mushrooms have a low total fat content in addition to high protein and carbohydrate content. These amounts usually range between 2 and 6% of dry matter) [99.In] fact, in the

present work, the studied specie also revealed a low fat content with values 6.2 ± 0.5 g/100 g /dw.

The reported value of ash is also fairly low (0.0160 ± 0.0004g/100 g dw) in comparison with the moisture content which present a high rate (92.2 ± 0.6). The mineral content (ash) of mushrooms ranges between 5 and 12 percent dry matter, according to literature. Potassium and phosphorus are the most abundant elements in mushroom ash, with contents that are higher or equal to those found in most vegetables. Magnesium, calcium, and sodium, on the other hand, are usually present in smaller quantities .[99]

Low energetic values, with an average of around 431 kcal/100 g dw, result from the low fat content combined with the inclusion of partially digestible or indigestible carbohydrates, making mushrooms low calorie and nutritious foods excellent to be incorporated into low calorie diets .[99]

Soluble sugar composition

Complete sugars are flavor compounds found in mushrooms that contribute to the sweetness of the flavor .[100] As a result, the high sugar content will trigger a mild sweet taste perception. Table 21 shows the sugar content of the wild edible mushroom specie studied. Mannitol and trehalose were the main sugars detected in the studied mushroom; the concentrations ranged from 21 ± 1mg/100 g DW.

Table 21 : Sugar composition of *Tricholoma terreum* (Schaeff.) P.Kumm. (mean ± SD)

	Tricholoma terreum
Mannitol (g/100 g dw)	13.0 ± 0.9
Trehalose (g/100 g dw)	7.6 ± 0.2
Total sugars (g/100 g dw)	21 ± 1

We can see from the table that the rate of Mannitol (13 g/100 g dw) is greater than Trehalose (7.6 g/100 g dw).

According to literature, those values appeared to be related to the contents of soluble non-structural polysaccharides. The aqueous extracts of *T. terreum* are much viscous and

gelatinous. Nevertheless, these values are within the range of those reported by Coli et al. (1988).Some works report the presence of mannitol pointing in *T.terreum*, this polyol as responsible for his bioactive properties, such as diuretic, anti-tussive and anti-free radical activities .[101]

Fatty acids profile

The results for the fatty acids composition of the studied edible mushrooms are presented in Table 22:

Table 22 : Fatty acids profile of *Tricholoma terreum* (Schaeff.) P. Kumm. (Mean ± SD)

	Tricholoma terreum
C16:0	10.0 ± 0.3
C18:0	2.19 ± 0.07
C18:1n9	55 ± 2
C18:2n6	31 ± 2
Total SFA (% of total FA)	12.2 ± 0.2
Total MUFA (% of total FA)	55 ± 2
Total PUFA (% of total FA)	31 ± 2

Palmitic acid (C16:0); stearic acid (C18:0); oleic acid (C18:1n9); linoleic acid (C18:2n6). SFA- saturated fatty acids; MUFA- monounsaturated fatty acids; PUFA- polyunsaturated fatty acids.

In addition to these fatty acids, 13 more fatty acids were also detected, but in an individual percentage of less than 1%, hence they were not considered in the Table 22. The fatty acids found were: caproic acid (C6:0); caprylic acid (C8:0); capric acid (C10:0); lauric acid (C12:0); myristic acid (C14:0); pentadecanoic acid (C15:0); palmitoleic acid (C16:1); heptadecanoic acid (C17:0); α-linolenic acid (C18:3n3); cis-11,14-eicosadienoic acid (C20:2);cis-5,8,11,14,17-eicosapentaenoic acid and erucic acid (C20:5n3+ C22:1n9);lignoceric acid (C24:0); and nervonic acid (C24:1).

The main fatty acids found in mushrooms are the saturated palmitic and stearic acids (C16:0), (C18:0), the unsaturated oleic acid (C18:1n9), and predominantly the

polyunsaturated linoleic acid (C18:2n6). Mushrooms are quite rich in unsaturated fatty acids, being mushrooms a source of essential fatty acids.

The unsaturated fatty acids usually prevailed over saturated fat [97]these were the majority fatty acids found in the studied species, with the exception of *P. linteus*, since the percentage of SFA (58.3%) was the highest fraction comparing with MUFA (14.6%) and PUFA (27.1%), similar results was also reported in on *P. linteus* from China.[101]

These results are similar to those reported in turkey,the amount of MUFA was found at the highest value and the linoleic and oleic acids percentages for *T.terreum* are dominant. The unsaturated fatty acid/saturated fatty acid ratio is 3.73 for *T. terrum*.[102]

5. CONCLUSION AND FUTURE PERSPECTIVES

The present work aimed to analyze and evaluate the production of phenolic and volatiles compounds (secondary metabolites) in samples of one of ectomycorrhizal fungi, *P. involutus*, isolated and in symbiosis with the roots of *C. sativa* to see if this initial symbiosis phases constitute a condition of oxidative stress that will trigger the defense of fungi hence their elicitation to produce secondary metabolites.

Regarding the phenolic compound's profiles, the same phenolic compounds were found in both roots control and roots in co-culture with *P. involutus* for 24h and 48h.

We can conclude that when in symbiosis for a short mycorrhizal time, the catching effect of the roots of *C. sativa* is the same compared to the effect observed when growing alone.

Regarding the fungus *P. involutus*, we found fragments of compounds that should be present in the three samples (control, co-culture 24h and 48h), namely acids (phenylethanol).

The other goal of this study was the characterizing of different bioactive compounds in different mushrooms and further evaluating their bioactivity. Additionally, the nutritional value was also evaluated for *T. terreum.*

Regarding the phenolic compound's profiles of edible mushrooms, all the samples (*T. terreum*, *T. equestre*, *B. pinicola* 2 and 3, and *L. deliciosus*) presented 2 phenolic acids (protocatechuic acid, *p*-hydroxybenzoic acid).

For other organic acids, five organic acids were identified, namely oxalic acid, quinic acid, malic acid, citric acid, and fumaric acid (the last in trace amounts).

The hydromethanolic extracts of *B. pinicola* 3 showed the highest antioxidant activity, and anti-microbial activity. Comparing the studied mushrooms, *B. pinicola* 3 proved to be the best source of the surveyed bioactive compounds

For the growth of isolated mushrooms in vitro, we can conclude that this was highly dependet on the culture media (MMN or PDA).

Regarding the nutritional parameters, carbohydrates were the main macronutrient present in the species and the rate of mannitol was greater than trehalose.

The studied edible mushrooms are quite rich in unsaturated fatty acids, namely oleic acid (C18:1n9), the most abundant fatty acid.

In general, *T. terreum* proved to be a potential pharmaceutical alternative, owing to the nutritional value and a valuable source of bioactive compounds, especially fatty and organic acids.

As future perspectives, the mycorrhization process still has some points to clarify. The compounds and reactions involved during this symbiosis must be studied, both from an ecological perspective and a biotechnological perspective. Furthermore, in vitro culture emerges as a process for obtaining species and compounds of interest that should be explored.

Finally, as mushrooms are an excellent source of interesting bioactive compounds, they can and should be explored as alternative sources for the development of pharmaceutical/nutraceutical or cosmetic formulations.

6. REFERENCES

1. Brundrett M. Diversity and classification of mycorrhizal associations. *Biol Rev Camb Philos Soc*. 2004;79(3):473-495.

2. R. Hanson the Chemistry of Fungi (Department of Chemistry, University of Sussex, and Brighton, UK).2008; 136(7): 978-0-85404.

3. Liu YJ, Hodson MC, Hall BD. Loss of the flagellum happened only once in the fungal lineage : phylogenetic structure of Kingdom Fungi inferred from RNA polymerase II subunit genes. 2006;13:1-13.

4. Hawksworth DL, Lücking R. Fungal Diversity Revisited : 2, 2 to 3, 8 Million Species. 2017.

5. Heijden MGA Van Der, Martin FM, Sanders IR. Tansley review Mycorrhizal ecology and evolution : the past, the present, and the future. 2015:1406-1423.

6. Stamets P. *Growing Gourmet and Medicinal Mushrooms*. 3rd Editio. California: Ten Speed Press; 2000.

7. Hanson JR. *The Chemistry of Fungi*. Department of Chemistry, University of Sussex, Brighton, UK: The Royal Society of Chemistry; 2008.

8. Plant science 4U .Difference between ectomycorrhizae and endomycorrhizae. 2013.

9. Sally S, Read D. *Mycorrhizal Symbiosis*. 3rd Editio. (Press A, ed.). Australia: Science Direct; 2008.

10. Agarwal, P. and Sah, P. Ecological importance of ectomycorrhizae in world forest ecosystems.Nature and Science.2008; 7(2):107-116.

11. Michael H.b,Hayes, Rosalen Mylatte ,Roger S swift . Chapter 2 Humin : Its composition and importance in soil organic matter ; Advances in agronomy. 2017;143:47-138.

12. Sally S, Read D. *Mycorrhizal Symbiosis*. 3rd Editio. (Press A, ed.). Australia: Science Direct; 2008.

13. Agarwal, P. and Sah, P. Ecological importance of ectomycorrhizae in world forest ecosystems.Nature and Science.2008; 7(2):107-116

14. Michael H.b,Hayes, Rosalen Mylatte ,Roger S swift . Chapter 2 Humin : Its composition and importance in soil organic matter ; Advances in agronomy. 2017;143:47-138.

15. Abarenkov K, Tedersoo L, Nilsson R. H, Vellak K, Saar I, VeldreV,et al. .PlutoFa web based workbench for ecological and taxonomic research, with an online implementation for fungal ITS sequences.*Evol. Bioinform.*10.4137/EBO.S627.2010*;* 6:189-196.

16. Journal of Experimental Botany.59: 1115–1126,https://doi.org/10.1093/jxb/ern059

17. Martins A, Z.A. Siddiqui et al (Eds.). *In vitro* Mycorrhization of Micropropagated Plants: Studies on *Castanea sativa* Mill. Mycorrhizae:Sustainable Agriculture and Forestry. Springer, Dordrecht, the Netherlands. 2008; 321-336.

18. Scannerini, S, Smith, D., Bonfante-Fasolo, P., Gianinazzi-Pearson**,** V. (Eds.). Host fungs interaction in ectomycorrhizae.Silvano scannerini et al ., :*cell to cell signals in Plant ,Animal and microbial symbiosis* .1988; 17:1010-8793.

19. S.Declerck, D.G.Strullu, J.A.Fortin .Sebacinaceae: Culturable Mycorrhiza-Like Endosymbiotic Fungi and Their Interaction with Non-Transformed and Transformed Roots. *In Vitro Culture of Mycorrhizas(soil biology)*.2005; 291-312.

20. Rai.M.Current advances in mycorrhization in micropropagation. *In vitro* cellular developmental biology-Plant (society for *in vitro* biology).2001; 37(2):158-16.

21. Alizoti. P.G, and Aravanopoulos, F.A.Geneticvariationof fruit traits inHellenic chestnut (*Castanea sativa* Mill.) populations: a first assessment. ActaHortic. 2005; 693: 413–420.

22. INIAV I.P. ECONOMIC IMPORTANCE. https://projects.iniav.pt/NewCastRootstocks/index.php/en/the-chestnut/economic-importance. Published 2018.

23. Borges O, Gonçalves B, Carvalho J, Correia P, Silva A. Nutritional quality of chestnut (*Castanea sativa* Mill.) cultivars from Portugal. *Food Chem*. 2008;106:976-984.

24. Baptista P, Martins A, Tavares R, Lino-Neto T. Diversity and fruiting pattern of macrofungi associated with chestnut (Castanea *sativa*) in the Tr??s-os-Montes region (Northeast Portugal). *Fungal Ecol - FUNGAL ECOL*. 2010;3:9-19.

25. Reis F, Tavares R, Baptista P, Lino-Neto T. Mycorrhization of Fagaceae Forests Within Mediterranean Ecosystems. In: *Mycorrhiza - Function, Diversity, State of the Art: Fourth Edition*. ; 2017:75-97.

26. N.A.M.Saleh, in Encyclopedia of Food Sciences and Nutrition (Second Edition), 2003.

27. Mag P, Karwasara VS, Tomar P, Dixit VK. Influence of fungal elicitation on glycyrrhizin production in transformed cell cultures of Abrus precatorius Linn. 2011:307-314.

28. Z. Angelova, S. Georgiev& W. Roos. Elicitation of Plants, Biotechnology & Biotechnological Equipment.2006; 20(2): 72-83. Link to this article: https://doi.org/10.1080/13102818.2006.10817345

29. Zhao J, Davis LC, Verpoorte R. Elicitor signal transduction leading to production of plant secondary metabolites.Biotechnologie Advances. 2005;23(4):283-333.

30. Gadzovska S, Maury S, Delaunay A, Spasenoski M, Joseph C, Hagège D. Jasmonic acid elicitation of Hypericum perforatum L. cell suspensions and effects on the production of phenylpropanoids and naphtodianthrones. *Plant Cell Tissue Organ Cult*. 2007;89(1):1-13.

31. Verpoorte, R. and Alfermann, A.W. Metabolic engineering of plant secondary metabolism. Dordrecht, the Netherlands: Kluwer Academic Publishers.2000; 9: 323-343

32. Wuyts, N., De waele, D. and Swennen, R. Extraction and partial characteriza- -tion of polyphenol oxidase from banana (Musa acuminate grandrnaine) roots. Plant PhysiolBiochem. 2006; 44:308-314.

33. M. Herrero, M. Plaza, A. Cifuentes, E. Ibáñez. Phenolic compounds can be defined as plant substances. Which process in common an aromatic ring bearing one or more hydroxyl groups. In liquid chromatography (second Edition).2017.

34. .Loomis, W. D. and Battaile, J., Plant phenolic compounds and the isolation of plant enzymes, phytochemistry.1966; 5:423.

35. Chandrasekara and Joseph Kumar, Roots and Tuber crops as functional foods .A Review on phytochemical constituents and their potential health benefits .2016; 15 : https://doi.org/10.1155/2016/3631647

36. .Michalak A. Phenolic Compounds and Their Antioxidant Activity in Plants Growing under Heavy Metal Stress. Pol J Environ Stud 2006; 15:523-530

37. Agrawal, A. A. Macroevolution of plant defense strategies. TRENDS in Ecology and Evolution.2006; 22:103-109.

38. Bate-Smith, E. C. Astringent tannins of Acer species. Phytochemistry, 1997; 16: 1421–1426.

39. Effah, Holopainen,JMc Cormick A.Potential roles of volatile organic compounds in plantcompetition, perspectives in plant Ecology, Evolution and systematic. 2019; 38:58-63.

40. E.Dudley, W.J.Griffiths, InAdvances in Protein Chemistry and Structural Biology. 2010; 80: 1-284.

41. Dudareva, N, Negre, F., Nagegowda, D. A. &Orlova, I. Plant volatiles: recent advances and future perspectives.*Critical reviews in plant sciences*.2006; 25: 417–440.

42. Peñuelas, J. &Llusia, J. The complexity of factors driving volatile organic compound emissions by plants.*Biologia Plantarum.*2001; 44: 481–487.

43. Natalia Dudareva1,2, Antje Klempien1, ‚Jo€elle K. Muhlemann1 and Ian Kaplan3 .Function and metabolic engineering of plant volatile organic compounds. Tansley review Biosynthesis.2013; 198:16-3244.

44. Arimura G, Ozawa R, Shimoda T, Nishioka T, Boland W, Takabayashi J. 2000. Herbivory-induced volatiles elicit defence genes in lima bean leaves. Nature 406:512–515.

45. .Hardege JD, Bartels-Hardege H, ZeeckE.Volatile compound from the celomic fluid of Nereissuccinea – biological activity as sex pheromone. Invertebr. Reprod. Dev. 1991; 19:83–85.

46. Dudareva, N. &Pichersky, E. Biochemical and molecular genetics aspects of floral scents.Plant physiology. 2000; 122:627–634.

47. Baldwin, I. T, Halitschke, R , Paschold, A, Von Dahl, C. C. & Preston, C. A. Volatile signaling in plant-plant interactions: “talking trees” in the genomics era.Science.2006; 311: 812–815.

48. Heil, M. &Karban, R. Explaining evolution of plant communication by airborne signals.Trends in ecology & evolution.2010; 25:137–144.

49. .Holopainen, J. K. &Gershenzon, J. Multiple stress factors and the emission of plant vocs.Trends in plant science. 2010; 15:176–184.

50. Abdul Rashid War, Michael Gabriel Paulraj, Tariq Ahmad, Abdul Ahad Buhroo, Barkat Hussain, Savarimuthu Ignacimuthu, Hari Chand Sharma. Mechanisms of plant defense against insect herbivores.Plant signaling &behavior.2012; 7:1306–1320.

51. Pinto S, Barros L, Sousa MJ, Ferreira ICFR. Chemical characterization and antioxidant properties of Lepista nuda fruiting bodies and mycelia obtained by in vitro culture : effects of collection habitat and culture media.

52. McGeer PL, McGeer EG. Inflammation, autotoxicity and Alzheimer disease. Neurobiol Aging. 2001;22(6):799-809.

53. Barros L, Cruz T, Baptista P, Estevinho LM, Ferreira ICFR. Wild and commercial mushrooms as source of nutrients and nutraceuticals. 2008;46:2742-2747.

54. Guzmán G, Cortés-pérez A, Guzmán-dávalos L, Ramírez-guillén F, Sánchez-jácome

MR. An emendation of Scleroderma , new records , and review of the known species in. Rev Mex Biodivers. 2013;84(1970):S173-S191.

55. Ruthes AC, Smiderle FR, Iacomini M. Mushroom heteropolysaccharides : A review on their sources , structure and biological effects. Carbohydr Polym. 2016;136:358-375.

56. A Technique for Mycelial Development of Ectomycorrhizal Fungi on Agar Media. 2002;1.

57. Murasnige T. A Revised Medium for Rapid Growth and Bio Agsays with Tohaoco Tissue Cultures. 1962;15.

58. Osterc G, Fras MZ, Vodenik T, Luthar Z. The propagation of chestnut (*Castanea sativa* Mill .) nodal explants. 2005;411-418.

59. Moro et al. Anti-inflammatory activity of methanolic extracts from edible mushrooms in LPS activated RAW 264.7 macrophages.Foodchemistry.2012: 350-355

60. Chemical Characterization and Antioxidant Properties of Three Wild Edible Mushroom Species from Northeastern Portugal. Food Analytical Methods .2012; 7: 645–652.

61. Lillian Barros, Montserrat Dueñas, Isabel C.F.R. Ferreira, Paula Baptista, Celestino Santos-Buelga: Phenolic acids determination by HPLC–DAD–ESI/MS in sixteen different Portuguese wild mushrooms species. Food and Chemical Toxicology. 2009; 47(6):1076-1079.

62. Oludemi Taofiq, Ricardo C. Calhelha, Sandrina Heleno, Lillian Barros, Anabela Martins, Celestino Santos-Buelga, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira: The contribution of phenolic acids to the anti-inflammatory activity of mushrooms: Screening in phenolic extracts, individual parent molecules and synthesized glucuronated and methylated derivatives. Food Research International.2015; 76(3):821-827.

63. Yuswan MH, Al-Obaidi JR, Rahayu A, Sahidan S, Shazrul F, Fauzi D .New bioactive molecules with potential antioxidant activityfrom various extracts of wild edible Gelam mushroom (Boletusspp.). AdvBiosciBiotechnol.2015; 06(04):320–329.

64. Carocho, M., Ferreira, I.C.F.R.. A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives. Food Chem. Toxicol. 2013 ;51 :15–25.

65. Goetz, M.E., Luch, A. Reactive species: A cell damaging rout assisting to chemical carcinogens. Cancer Lett. 2008;266:73-83.

66. Sliva, D.,. Medicinal mushroom Phellinus linteus as an alternative cancer therapy. Experimental and Therapeutic Medicine. 2010; 1:407-411.

67. Saxena P, et al.,. Role of phosphorylation of tyrosine dephosphorylation in the control of the copy number of the 2-micron circle of the yeast plasmid. Cell Mol Biol Res 1994; 40:215-22.

68. Alves, M., Ferreira, I.F.R., Dias, J., Teixeira, V., Martins, A., Pintado, M., A review on antimicrobial activity of mushroom (basidiomycetes) extracts and isolated compounds. Planta Med.2012; 78 :1707–1718

69. F. Badria, H. Fathy, A. Fatehe, D. Elimam, and M. Ghazy, "Evaluate the cytotoxic activity of honey, propolis, and bee venom from different localities in Egypt against liver, breast, and colorectal cancer," Journal of Apitherapy.2017;2:1–8.View at: Publisher Site | Google Scholar

70. L. J. McGaw, E. E. Elgorashi, and J. N. Eloff, "Cytotoxicity of African medicinal plants against normal animal and human cells," Toxicological Survey of African Medicinal Plants.2014;8:181–233.View at: Publisher Site | Google Scholar

71. Lockowandt, L., Pinela, J., Roriz, C.L., Pereira, C., Abreu, R.M.V., Calhelha, R.C., Alves, M.J., Barros, L., Bredol, M., Ferreira, I.C.F.R. Chemical features and bioactivities of cornflower (Centaurea cyanus L.) capitula: The blue flowers and the unexplored non-edible part. Ind. Crops Prod.2019; 128:496–503.

72. LillianBarros, Lillian Barros, AnabelaMartins, Isabel C.F.R.Ferreira: Towards chemical and nutritional inventory of Portuguese wild edible mushrooms in different habitats. Food Chemistry. 2012; 130(2):394-403

73. Prior, R.L., Wu, X., Schaich, K.Standardized methods for the determination ofantioxidant capacity and phenolics in foods and dietary polyphenols as determined by amodified ferric reducing/antioxidant power assay. Journal of Agricultural and Food Chemistry.2005; 53: 4290-4302.

74. Lock, K., Smith, R.D., Dangour, A.D., Keogh-Brown, M., Pigatto, G., Hawkes, C., Fisberg, R.M., Chalabi, Z. Health, agricultural, and economic effects of adoption of healthy diet recommendations. The Lancet. 2009; 376:1699-1709.

75. Vasconcelos MCBM, Bennett RN, Quideau S, Jacquet R, Rosa EAS, Ferreira-Cardoso JV.Centre for the Research and Technology of Agro-Environment and Biological Sciences, Integrative Biology & Quality Area, Universidade de Trás-os-Montes e Alto Douro (UTAD), Apartado.2009; 1013:5000-801 Vila Real, Portugal.

76. Heleno, S.A, Barros, L., Martins, A, Queiroz, M.J.R.P, Morales, P, Fernández-Ruiz, V, Ferreira, I.C.F.R. Chemical composition, antioxidant activity and bioaccessibility studies in phenolic extracts of two Hericium wild edible species. LWT - Food Sci. Technol. 2015; 63:475–481.

77. Barros, L, Calhelha, R. C., Queiroz, M. J. R. P, Santos-Buelga, C., Santos, E. A., Regis, W. C. B., & Ferreira, I. C. F. R.. The powerful in vitro bioactivity of Euterpe oleracea Mart. seeds and related phenolic compounds. Ind. Crop Prod.2015;76 :318–322.

78. Puttaraju, N, Venkateshaiah, S. U., Dharmesh, S. M, Urs, S. M, & Somasundaram, R. Antioxidant activity of indigenous edible mushrooms. Journal of Agricultural and Food Chemistry.2006; 54:9764–9772.

79. Alves, G., Lamelo, M., Álvarez-García, P., Velasco, M., Delgado, A., Horcajada, J.P., et al. Impact of empirical treatment in extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella spp. bacteremia. A multicentric cohort study. BMC Infectious Diseases.2012;12,245.

80. Choe, E., & Min, D.B. Mechanisms of antioxidants in the oxidation of foods. Comprehensive Reviews in Food Science and Food Safety. 2009; 8:345-358.

81. Altmeyer, P.J., Mattlies, U., Pawlak, F., Hoffmann, K., Frosch, P.J., Ruppert, P., Wassilew,S.W., Horn, T., Kreysel, H.W., Lutz, G., Barth, J., Rietzschel, I, Joshi, R.K. .Antipsoriatic effect of fumaric acid derivatives: results of a multicenter double-blind.1994; 8:200-225.

82. Raybaudi-Massilia, R.M., Mosqueda-Melgar, J., Martin-Belloso, O. Antimicrobial activity of malic acid against Listeria monocytogenes, Salmonella Enteritidis and Escherichia coli O157:H7 in apple, pear and melon juices. Food Control2009; 20:105-112.

83. Hanson, J.R. The chemistry of fungi. Royal Society of Chemistry: United Kingdom.2008.

84. Ribeiro, B., Rangel, J., Valentão, P., Baptista, P., Seabra, R.M., Andrade, P.B. .Contents of carboxylic acids and two phenolics and antioxidant activity of dried Portuguese wild edible mushrooms. Journal of Agricultural and Food Chemistry.2006; 54:8530-8537.

85. Küçükkurt D., Sarbatly R, Nithyanandam R .A review of the antioxidant potential of medicinal plant species. Food Bioprod Process. In press.2010.

86. Ferreira, I.C.F.R., Barros, L., Abreu, R.M.V. Antioxidants in wild mushrooms. Current Medicinal Chemistry.2009; 16:1543-1560.

87. Alves, M.J., Ferreira, I.C.F.R., Dias, J., Teixeira, V., Martins, A., Pintado, M. A review on antimicrobial activity of mushroom (Basidiomycetes) extracts and isolated compounds. Planta Medica.2012; 78:1707-1718.

88. Ferreira, I.C.F.R., Barros, L., Abreu, R.M.V. (2009). Antioxidants in wild mushrooms. Current Medicinal Chemistry.2009; 16:1543-1560.

89. Moro, C., Palacios, I., Lozano, M., D'Arrigo, M., Guillamón, E., Villares, A., Martínez, J. A., & García-Lafuente, A. Anti-inflammatory activity of methanolic extracts from edible mushrooms in LPS activated RAW 264.7 macrophages. Food Chemistry.2012; 130:350–355.

90. Kalač, A review of chemical composition and nutritional value of wild-growing and cultivated mushrooms. 2012;200–250,

91. Asgar, M.A., Fazilah, A., Huda, N., Bhat, R., Karim, A.A. Nonmeat protein alternatives as meat extenders and meat analogs. Comprehensive Reviews in Food Science and Food Safety.2010; 9: 513-529

92. Kalač, P. Chemical composition and nutritional value of European species of wild growing mushrooms: A review. Food Chemistry.2013; 113: 9-16.

93. Kalač, P. A review of chemical composition and nutritional value of wild-growing and cultivated mushrooms. Journal of the Science of Food and Agriculture.2009; 93: 209-218.

94. Lindequist, U., Niedermeyer, T.H., Jülich, W.D. The pharmacological potential of mushrooms. Evidence-Based Complementary and Alternative Medicine2005;2:285-299.

95. Das, S.K., Masuda, M., Sakurai, A., Sakakibara, M. Medicinal uses of the mushroom Cordyceps militaris: Current state and prospects. Fitoterapia.2010; 81: 961-968.

96. Deng, K., Zhang, Y., Ren, Z., Xie, L., Peng, W., Gan, B. Simultaneous determination of five fatty acids in Phellinus sp. by high-performance liquid chromatography with photodiode-array detection. Journal of Medicinal Plants Research, 2011; 5:2816-2821.

97. Ding, S., Chi, M.M., Scull, B.P., Rigby, R., Schwerbrock, N.M.J., Magness, S., Jobin, C., Lund, P.K. High-fat diet: Bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse. 2010; 5: e12-191.

98. Longvah, T., & Deosthale, Y.G. Compositional and nutritional studies on edible wild mushroom from northeast India. 1998.

99. Lyle, K.L. Complete guide to edible wild plants, mushrooms, fruits, and nuts: How to

find, identify, and cook them. Rowman & Littlefield: Maryland. Food Chemistry.2010; 63:331-334.

100. Mandal, S.M., Chakraborty, D., Dey, S. Phenolic acids act as signaling molecules in plant-microbe symbioses. Plant Signal & Behavior.2010; 5:359-368.

101. Mani-Lopez, E., García, H.S., López-Malo, A. Organic acids as antimicrobials to control Salmonella in meat and poultry products. Food Research International. 2012; 45: 713- 721.

102. Martins, N., Barros, L., Henriques, M., Silva, S., Ferreira, I.C.F.R. Activity of phenolic compounds from plant origin against Candida species. Industrial Crops and s and Products.2015; 74:648-670.