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Evaluation of the anticancer potential of the macrofungus *Pisolithus arhizus*

Graça Germana Santos da Costa

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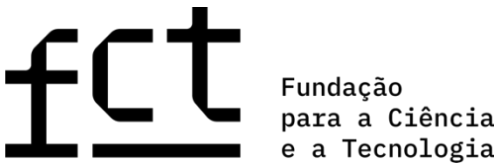

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Resumo

Os macrofungos produzem produtos bioativos, que podem contribuir significativamente para a saúde. *Pisolithus arhizus* é uma espécie de fungo pertencente ao filo Basidiomycota. É encontrado principalmente em associação com raízes de algumas árvores, estabelecendo uma relação mutualística com elas, facilitando a absorção de nutrientes do solo em troca de hidratos de carbono produzidos pela planta hospedeira. Este macrofungo tem despertado grande interesse devido às suas promissoras propriedades terapêuticas e efeitos bioativos.

Este trabalho teve como objetivo a avaliação do potencial anticancerígeno de um extrato de diclorometano:metanol (2:1). Extratos brutos foram preparados a partir do micélio e do meio de cultura de *P. arhizus*. Os extratos foram posteriormente separados em 9 frações recorrendo a cromatografia líquida de vácuo. As frações foram testadas quanto à citotoxicidade contra as linhagens celulares do adenocarcinoma do cólon RKO, neuroblastoma humano SH-SY5Y e fibroblastos 3T3-L1. A viabilidade celular foi avaliada através do ensaio de redução do brometo de 3-(4,5-dimetil-2-tiazolil)-2,5-difenil-2H-tetrazólio (MTT). Os resultados revelaram citotoxicidade contra a linhagem celular RKO em várias frações, com pouca ou nenhuma atividade nas células 3T3-L1. Os resultados obtidos apoiam conclusões previas de que *P. arhizus* é uma fonte promissora de compostos com atividade anticancerígena.

No entanto, estudos mais aprofundados são necessários para analisar os compostos químicos provenientes das várias partes de *P. arhizus* e investigar as suas atividades biológicas e toxicológicas. Explorar o potencial bioativo de *P. arhizus* é fundamental para desenvolver uma compreensão completa dos seus benefícios terapêuticos.

Palavras-chave: *Pisolithus arhizus;* macrofungo; potencial anticancerígeno; compostos naturais

Abstract

Macrofungi produce bioactive compounds that can greatly enhance health. *Pisolithus arhizus* is a species of fungus belonging to the phylum Basidiomycota. It is mainly found in association with the roots of some trees, establishing a mutualistic relationship with them, facilitating the absorption of nutrients from the soil in exchange for carbohydrates produced by the host plant. This macrofungus has aroused great interest due to its promising therapeutic properties and bioactive effects.

This work aimed to evaluate the anticancer potential of a dichloromethane:methanol (2:1) extract. Crude extracts were prepared from the mycelium and culture medium of *P. arhizus*. The extracts were subsequently separated into 9 fractions using vacuum liquid chromatography. The fractions were tested for cytotoxicity against the colon adenocarcinoma cell lines RKO, human neuroblastoma SH-SY5Y and 3T3-L1 fibroblasts. Cell viability was assessed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay. The results revealed cytotoxicity against the RKO cell line in several fractions, with little or no activity in 3T3-L1 cells. The obtained results support previous conclusions that *P. arhizus* is a promising source of compounds with anticancer activity.

However, more in-depth studies are needed to analyze the chemical compounds originating from the various parts of *P. arhizus* and investigate their biological and toxicological activities. Exploring the bioactive potential of *P. arhizus* is critical to developing a complete understanding of its therapeutic benefits.

Keywords: *Pisolithus arhizus;* macrofungus; anticancer potential; natural compounds

Table of Contents

1. General introduction 1
1.1. Macrofungi 5
1.2. *Pisolithus arhizus* 8
1.2.1. Characteristics 8
1.2.2. Bioactive potential 10
2. Aim of the dissertation 20
3. Materials and Methods 21
3.1. *Pisolithus arhizus* 21
3.2. Extraction 22
3.3. Fractionation 22
3.3.1. Sub-Fractionation of fraction C of the mycelium 24
3.4. High-Performance Liquid Chromatography (HPLC) 25
3.5. Cytotoxicity assay 25
3.5.1. Cell cultures 25
3.5.2. Cell passages 26
3.5.3. Cell counting 26
3.5.4. Cell viability assay 26
3.5.5. Dose response assay 27
3.6. Statistical Analysis 28
4. Results 29
4.1. Extraction and fractionation results 29
4.2. Sub-fractionation results 30
4.3. High-Performance Liquid Chromatography (HPLC) 30
4.4. Cytotoxic activity of the fractions 31
4.4.1. 3T3-L1 cell line 31
4.4.2. RKO cell line 35
4.4.3. SHSY5Y cell line 38
5. Discussion 39
6. Conclusion 47
7. Future perspectives 48

References 49
Appendices 72

List of Abbreviations

3T3-L1 – Embryonic mouse fibroblast cell line

ABTS – 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ATCC – American Type Culture Collection

DCM – Dichloromethane

DMEM – Dulbecco's modified Eagle's medium

DMSO – Dimethyl sulfoxide

DPPH – 2,2-di(4-tertoctylphenyl)-1-picrylhydrazyl

EtOAc – Ethyl acetate

FBS – Fetal bovine serum

Hex – Hexane

HPLC – High-performance liquid chromatography

FRAP – ferric reducing antioxidant power

MeOH – Methanol

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide NAD(P)H – Nicotinamide adenine dinucleotide phosphate

OD – Optical density

PBS – Phosphate-buffered saline

PDA – Potato Dextrose Agar

RKO – Colon adenocarcinoma cell line

SH-SY5Y – Human neuroblastoma cell line

VLC – Vacuum Liquid Chromatography

List of Tables

Table 1. Compounds and extracts of Pisolithus arhizus with anticancer activity. 13
Table 2. Extracts of Pisolithus arhizus with antimicrobial activity. 17
Table 3. Compounds and extracts of Pisolithus arhizus with antioxidant and immunosuppressive activity..... 19
Table 4. Eluents for the Vacuum Liquid Chromatography fractionation procedure of the medium and mycelium. 24
Table 5. Cell lines used in the cytotoxicity assays, origin and recommended cell density.....25
Table 6. Volume of solvent used and dried extracts recovered after the extraction process.29
Table 7. Weight of the fractions resulted from the fractionation of the medium and mycelium crude extracts. 29
Table 8. Fraction obtained with each tube and mass recovered from fraction C of the mycelium. 30
Table 9. Weight of the extract from subfractions of the fraction 2+3 from sub-fractionation of fraction C of the mycelium. 31

List of Figures

Figure 1. Estimated proportions of living organisms on Earth. Retrieved from (Cheng, 2022)[2]. 1

Figure 2. Cross-section of a mushroom sporocarp, showing the mycelium and the hyphae. Retrieved from (STOWA, 2020)[12]. 2

Figure 3. Mycorrhizal network exchanges information and nutrients in between trees. Retrieved from (How trees trade with each other below ground, New Zealand Geographic)[18]. 3

Figure 4. *Pisolithus arhizus.* 8

Figure 5. Chemical structure of pisosterol 11

Figure 6. Chemical structures of pisolithin A (a) and pisolithin B (b). 15

Figure 7. Chemical structure of 5-hexadecenoic acid. 15

Figure 8. Chemical structures of the triterpenoids pisolactone (a) and 7,22-dien-3-ol, 24-methyl lanostane (b). 16

Figure 9. Isolated mycelium on PDA medium. Retrieved from Lima, (2023)[136]. 21

Figure 10. Plastic cups with the mycelium collected. Retrieved from Lima, (2023)[136] 21

Figure 11. Schematic diagram of the extraction process. Created in BioRender.com. 22

Figure 12. Vacuum Liquid Chromatography system. 23

Figure 13. Nine fractions, ranging from A (lowest polarity) to I (highest polarity). Created in BioRender.com. 23

Figure 14. 96 well plate scheme for the cell viability assays. Created in BioRender.com. 27

Figure 15. Chromatographic profile of the fraction 2+3 from sub-fractionation of fraction C of the mycelium. 30

Figure 16. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the 3T3-L1 cell line on medium, at timepoints t=24 h and t=48 h. 32

Figure 17. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the 3T3-L1 cell line on mycelium, at timepoints t=24 h and t=48 h. 32

Figure 18. Effect of each concentration on the viability of the 3T3-L1 cell line on fraction B of the mycelium, at timepoints t=24 h and t=48 h. 33

Figure 19. Effect of each concentration on the viability of the 3T3-L1 cell line on fraction C of the mycelium, at timepoints t=24 h and t=48 h. 33

Figure 20. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the 3T3-L1 cell line on sub-fractions of fraction C of the mycelium, at timepoints t=24 h and t=48 h... 34
Figure 21. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the 3T3-L1 cell line on sub-fractions 2+3 of fraction C of the mycelium, at timepoints t=24 h and t=48 h. 34
Figure 22. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the RKO cell line on medium, at timepoints t=24 h and t=48 h..... 35
Figure 23. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the RKO cell line on mycelium, at timepoints t=24 h and t=48 h. 35
Figure 24. Effect of each concentration on the viability of the RKO cell line on fraction B of the mycelium, at timepoints t=24 h and t=48 h. 36
Figure 25. Effect of each concentration on the viability of the RKO cell line on fraction C of the mycelium, at timepoints t=24 h and t=48 h. 36
Figure 26. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the RKO cell line on sub-fractions of fraction C of the mycelium, at timepoints t=24 h and t=48 h. 37
Figure 27. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the RKO cell line on sub-fractions 2+3 of fraction C of the mycelium, at timepoints t=24 h and t=48 h. 37
Figure 28. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the SHSY5Y cell line on sub-fractions 2+3 of fraction C of the mycelium, at timepoints t=24 h and t=48 h..... 38

1. General introduction

Fungi constitute the second largest group of organisms on Earth, surpassed solely by insects (Figure 1)[1,2]. These organisms have a worldwide distribution and thrive in a wide range of environments, including glaciers, deserts, tropical and temperate regions, as well as high latitude areas[3].

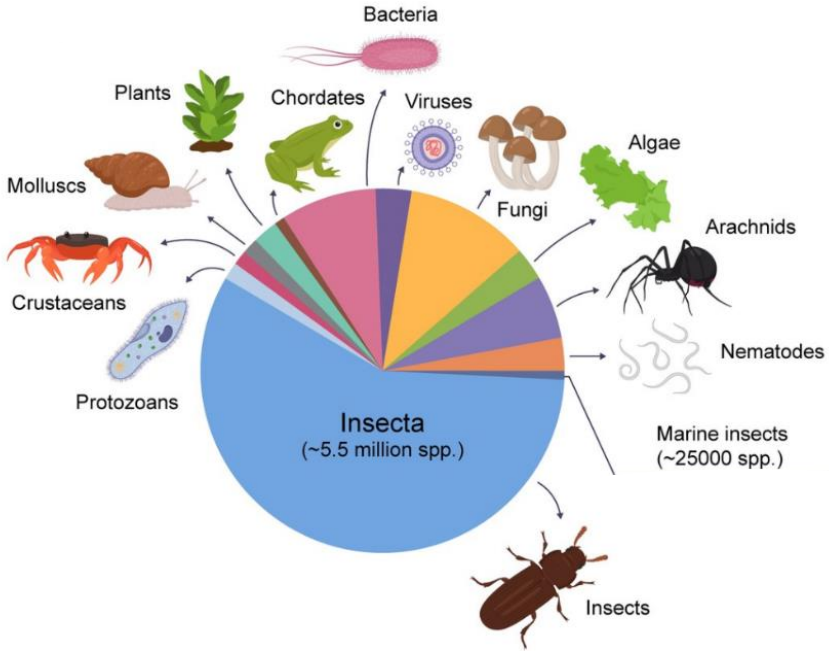

Figure 1. Estimated proportions of living organisms on Earth. Retrieved from (Cheng, 2022)[2].

Until 1969, fungi were classified as plants due to their appearance and lack of mobility, until Whittaker created the kingdom Fungi[4]. In 1998, T. Cavalier-Smith proposed a new taxonomic organization that divided the diversity of life into six distinct kingdoms: Animalia, Bacteria, Chromista, Fungi, Plantae, and Protozoa. In this new system, fungi were again recognized as a separate kingdom, although organisms had been removed from the original Fungi kingdom to other kingdoms, including Chromista[5].

More recently, following the consensus of most of the world's mycological community and using molecular biology tools, it was determined that the kingdom Fungi should be divided into 7 phyla, Ascomycota, Basidiomycota, Blastocladiomycota, Chytridiomycota, Glomeromycota, Neocallimastigomycota and Microsporidia, 10 subphyla, 35 classes, 12 subclasses and 129 orders[6].

Of the 1.5 to 5 million estimated species of fungi, only around 120,000 have been classified. This arises from the challenge associated with isolating and cultivating the majority of fungi,

which has also been a barrier to assigning their functions[7]. Due to significant progress in the use of molecular techniques to analyze the genetic material of microorganisms over the last two decades, some of these obstacles have been overcome, resulting in a notable increase in the relevance of fungi in various ecosystems[8,9].

Fungi exhibit remarkable variability, encompassing both macroscopic forms (like mushrooms, truffles, and puffballs) as well as microscopic organisms. Despite their diversity, they possess shared characteristics[10].

Fungi are heterotrophic organisms, and thus their metabolism relies on the organic and inorganic matter available in the ecosystem[11]. These organisms take nutrients from the environment by absorption (extracellular digestion or symbiosis), using structures called hyphae; long, filamentous and branched structures (Figure 2)[12]. The aggregation of these structures, the mycelium, plays a fundamental role in the recycling of organic matter and in the attachment of the fungus to the substrate[13,14].

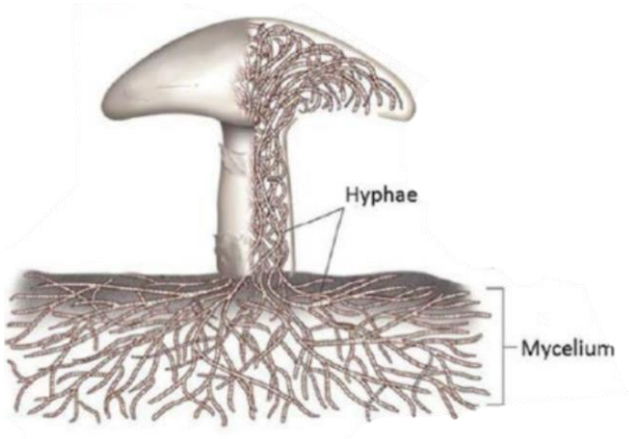

Figure 2. Cross-section of a mushroom sporocarp, showing the mycelium and the hyphae. Retrieved from (STOWA, 2020)[12].

Within the ecosystem, fungi build up different associations with other organisms, namely symbiosis. Here, there is an association between two or more organisms living in a close relationship, where they may benefit, be harmed, or not be affected by the association, called mutualism, parasitism and commensalism respectively[15]. Fungi can establish these relationships with animals, plants or even other fungi[16].

In nature, more than 90% of terrestrial plants form mycorrhizas[17]. These are symbiotic associations between plants and fungi (Figure 3)[18].

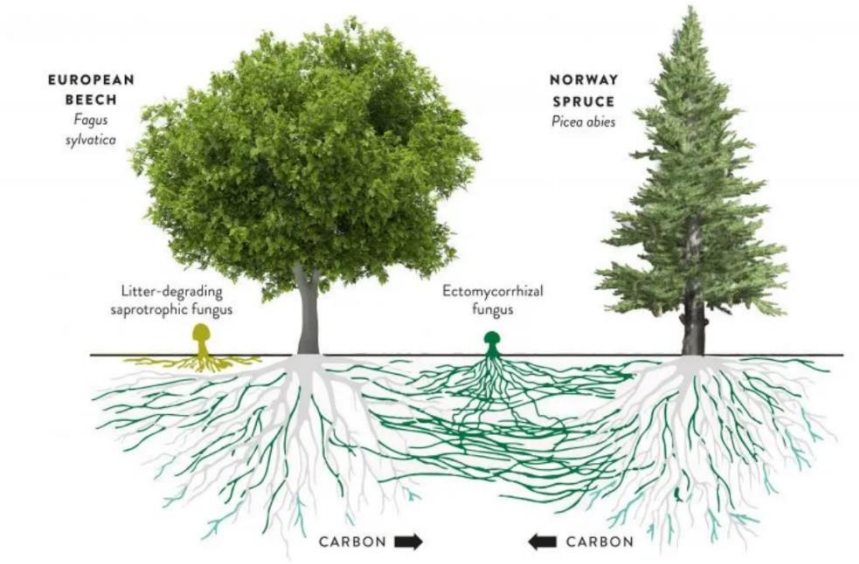

Figure 3. Mycorrhizal network exchanges information and nutrients in between trees. Retrieved from (How trees trade with each other below ground, New Zealand Geographic)[18].

In these symbiosis, both species involved benefit: the fungi, through their hyphae, supply mineral nutrients and water from the soil to the plant roots, while the plants, in return, transfer sugars produced by photosynthesis to the fungi[19].

The hyphae of the mycorrhizal fungi can extend their networks everywhere in the soil, thus allowing the mycorrhizal fungi to capture nutrients outside the area immediately around the root[20]. Mycorrhizal fungi play a fundamental role in the recycling of carbon (C), nitrogen (N) and phosphorus (P) in plant ecosystems, as well as other elements such as potassium (K) and sulfur (S)[21].

In natural ecosystems, plants obtain up to 80% of their nitrogen needs and up to 90% of their phosphorus needs from mycorrhizal fungi[22].

In addition, the state of health of the host plants can affect the growth of these fungi[23].

However, the average response of plants to mycorrhizal symbiosis apparently varies substantially between species, suggesting that evolutionary history may also exert an important influence on the existing variation in the degree of mutualism[24,25].

In general, mycorrhizal symbioses can be divided into several distinct association types, including arbuscular mycorrhizal and ectomycorrhizal, which differ in their evolutionary origins[26]. Although there is a single origin of arbuscular mycorrhizal symbiosis in plants and fungi, ectomycorrhizal symbiosis stems from multiple and independent evolutionary origins in plants and fungi[27,28]. Hoeksema et al. (2018) hypothesize that the different genetic origins and

environmental contexts of the independent evolutionary origins of the ectomycorrhizal symbiosis may have selected different strengths of this mutualism[25].

Fungi are responsible for most of the diseases that affect plants and are the cause of numerous diseases in humans[29]. They have also been exploited for their medicinal value for thousands of years[30]. In addition to their nutritional benefits, their ability to produce secondary metabolites makes them an important source of innovative chemistry, which has led to the development of several important pharmaceutical products that have been beneficial to the advancement of human civilization over time[31]. Fungal-based processes and fungal enzymes also play a key role in food and beverage production.

Fungi are recognized for their ability to produce a significant variety of compounds with bioactive properties such as antioxidants[32], anti-inflammatories and antivirals[33], as well as compounds that demonstrate the ability to reduce cholesterol, control diabetes[34] and autoimmune diseases, as well as activity against tumor growth[35,36].

Like plants, fungi accumulate a wide variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids[37]. Phenolic compounds include phenolic acids, flavonoids, hydroxybenzoic acids, lignins, tannins and oxidized polyphenols. These have biological activities, generally attributed to their antioxidant capacity. Antioxidant compounds have the important ability to eliminate free radicals and inhibit the oxidative mechanisms that lead to degenerative diseases[38,39].

Terpenoids such as sesquiterpenoids, diterpenoids and triterpenoids are the most commonly isolated metabolites of Basidiomycota[37]. Polysaccharides, alkaloids, proteins, fats, minerals, carotenoids, glycosides, terpenoids, folates, tocopherols, flavonoids, phenolics, volatile oils, ascorbic acid, lectins, enzymes and organic acids are all bioactive molecules synthesized by different fungal organisms[40,41].

The compact nature of fungal genomes renders them highly advantageous for genetic studies in eukaryotic biology and as efficient microbial cell factories in the fields of biotechnology and bioengineering[42,43].

1.1. Macrofungi

According to Chang and Miles (2004), macrofungi, such as mushrooms, puffballs and truffles, present a distinct fruiting body, which can be either epigean (above ground) or hypogean (underground), large enough to be observed with the naked eye and harvested by hand[44]. Macrofungi are distributed across diverse habitats, but they are particularly abundant in forests and meadows, where they play crucial roles in decomposition, nutrient cycling, and symbiotic relationships with plants[45].

Macrofungi have historically been considered a source of metabolites with bioactive potential[46]. These substances are basically divided into primary and secondary metabolites.

Primary metabolites relate to fundamental elements for the proper functioning of the organism, including sugars, amino acids and glycerol; they play a vital role in the development, maintenance and reproduction of living organisms[47]. Secondary metabolites are synthesized in the organism's final growth phase, typically during the stationary phase, and cover a wide range of compounds depending on the organism in question[48,49]. These metabolites are associated with biotechnological exploitation in the pharmaceutical industry's search for new antibiotics. Most of these compounds are obtained from fungi, such as penicillin, steroids or even cyclosporine[50].

In order to preserve the fruiting body from harmful organisms such as viruses, bacteria and insects, fungi have the ability to produce a variety of secondary metabolites, such as terpenoids and phenolics, which have antiviral, antioxidant and other biological activities, functioning as chemical defense mechanisms[51]. Nutraceuticals derived from macrofungi are obtained from mycelia and/or fruiting bodies and administered in the form of capsules, tablets or extracts[52].

Edible macrofungi represent a recognized but untapped source of precious foods and nutraceuticals that have the potential to promote human health and enhance quality of life[53]. However, inedible or poisonous species also offer a rich source of bioactive secondary metabolites, which can be used to develop mycopharmaceuticals, such as dietary supplements or nutraceuticals, with therapeutic properties and the potential to improve health[54,55].

Mushrooms represent one of the most recognizable groups of macrofungi. In the past, mushrooms were categorized as belonging to the group of so-called 'lower' plants in the

Division Thallophyta, as defined by Linnaeus. This classification was largely based on their relatively simple structures and anatomy devoid of complexities (lack of roots, stems, leaves, flowers and true seeds)[56].

More recent studies have established that the mushroom biota, along with other fungi, has enough distinct characteristics to justify the creation of an autonomous fungal kingdom, known as the Myceteae Kingdom[57].

Mushrooms can be divided into four categories: (1) those that are fleshy and edible fall into the category of edible mushrooms; (2) those that are recognized for their applications in medicine are called medicinal mushrooms; (3) those that are proven or suspected to be poisonous are called poisonous mushrooms; (4) a diverse category that houses numerous mushrooms with less defined properties is temporarily grouped together as 'other mushrooms'[58].

Although mushrooms have mainly been appreciated throughout history for their contribution to cooking and nutrition, there is also a long history of using certain mushrooms for their medicinal benefits and toning properties[59,60].

Mushrooms contain abundant active proteins, such as lectins with an antiviral effect[61], deoxyribonuclease with antitumor function[62] and ribotoxin with antiproliferative effect[63].

In general, mushrooms are approximately 90% water by weight, and the remaining 10% is divided into protein, carbohydrates, fiber and fat[64]. The mineral content is mainly potassium, calcium, phosphorus, magnesium, iron, zinc and copper[44]. Most mushrooms also contain vitamins such as niacin, thiamin, riboflavin, biotin and vitamin C, and a wide range of bioactive molecules such as terpenoids, steroids, phenols and nucleotides[65,66].

The content and type of biologically active substances can vary considerably in edible mushrooms; the concentrations of these substances are affected by differences in strain, substrate, cultivation, stage of development, age, storage conditions, processing and culinary practices[67,68].

Macrofungi seem to be particularly talented at producing unique terpenoids. One example is pleuromutilin, a tricyclic diterpene that serves as a natural antibiotic present in the mushroom cultures of *Clitopilus passeckerianus*[69]. Other notable examples include strobilurins (agrochemical fungicides) and illudins from *Lampteromyces* and *Omphalotus* species. Iludins are sesquiterpenes with an unusual cyclopropane and are considered promising sources for the development of potential anti-cancer drugs and agrochemical pesticides[70].

The use of mushrooms as a source of bioactive compounds has several advantages over plants. In addition, both mycelia and fruiting bodies can be cultivated quickly in liquid media. Finally, the growing medium can be manipulated to achieve desired concentrations of the active compounds, giving greater flexibility to the production process[71].
The use of mushroom-based products is considerably high due to the minimal regulation and safety compared to traditional medicines[72].

Truffles are a type of macrofungi that typically grows underground, often near the base of trees. Generally, truffles are firm, dense, and woody compared to the soft and fragile texture of mushrooms[73]. These macrofungi are ectomycorrhizal, meaning they form symbiotic root associations. In a complex life cycle, their mycelia establish symbiotic interactions primarily with the roots of various trees, including hazel, poplar, pine, eucalyptus, and oak[74].
Truffles, which thrive underground, develop spores within the sporocarp. However, truffle spores are not dispersed into the air. Instead, many truffles emit odors that attract mammals, prompting them to dig up and transport the truffles for consumption later. If the mammal drops or consumes only a portion of the truffle, the spores may scatter to a new location, facilitating the fungus's reproduction and propagation[75,76].
Truffles are gourmet mushrooms that are appreciated worldwide for their unique aromatic properties[77]. Their chemical compounds blend harmoniously with fatty substances like butter, cream, and cheeses[78,79].
In addition to their aromatic qualities, truffles boast a biochemical composition that offers numerous nutritional and medicinal benefits. They are abundant in essential nutrients such as carbohydrates, proteins, fats, minerals, lipids, and amino acids. Moreover, truffles are packed with phenolics, terpenoids, polysaccharides, and phytosterols[73,80,81].

Within puffballs, spores are generated internally, contained within the spherical structure of the fungus[82]. The puff of smoke emitted when a puffball is squeezed results from the release of spores through an opening of the ball[83].

1.2. *Pisolithus arhizus*

1.2.1. Characteristics

Pisolithus is a widely distributed genus of fungus that belongs to the family Sclerodermataceae and the phylum Basidiomycota[84].

The Basidiomycota phylum comprises around 40,000 classified species, representing approximately 25% of all described fungi[85,86]. The various properties of Basidiomycota play a key role in biotechnology and industry, providing essential benefits in fields such as enzyme production, bioremediation and pharmaceutical research[87,88]. The diversity of bioactive natural products derived from Basidiomycota is significant and includes compounds with various properties, including antimicrobial, anticancer, antioxidant, anti-inflammatory and nutraceutical activities, among others[89,90].

The structure of *Pisolithus arhizus* (syn. = *Pisolithus tinctorius*) (Figure 4), can be divided into two main regions: the sporocarp, a zone attached to the soil, with a hard and rigid texture; and an upper zone with soft tissues called pseudoperidoli, containing a brownish pigment and the spores of this fungus[91].

Figure 4. *Pisolithus arhizus.*

The characteristic structure of sexual reproduction in basidiomycetes (the name given to species of this phylum) is known as a basidium[85].

Typically, these fungi produce sexual spores externally – the basidiospores. The number of basidiospores produced by a single basidiocarp (fruiting body) can be extremely high[92].

Basidiospores can remain dormant and retain viability for several months or even years if conditions are unsuitable for germination. Dormancy is often exogenous in nature, meaning that the spores need some kind of external chemical or physical stimulus before germination can take place[93].

This fungus is widespread, growing in woods, orchards and arid soils. However, the production of basidiocarps seems to be more favorable in damp places and near roads[94,95].

This genus has globular fruiting bodies with diameters ranging from 3–14 cm, usually dark yellow or brown in color. The outer surface (peridium) is thin and smooth, often shiny, varying from yellow-brown to darker shades of purple[96].

The dehiscence (spontaneous opening due to the mushroom's maturity) is very irregular and the gleba is made up of numerous cavities (peridioles) where the spores are found encased in a black gelatinous matrix[11].

The rhizomorph (cluster of hyphae imitating roots) is diffuse and quite simple, making it easier to absorb water and nutrients. As the sporocarp matures, it gradually falls apart, releasing the spores. Fruiting takes place between fall and spring[96].

This genus also has the ability to form sclerotia, structures made up of the organization of parts of the mycelium with food reserves, which allow the fungus to survive during periods that are unfavorable for its development[95].

Pisolithus arhizus is an ectomycorrhizal fungus that lives in symbiosis with various species of trees and shrubs. Their filaments (hyphae) explore the soil and connect to the roots of trees, facilitating the transfer of water and nutrients from the soil, which are essential for their development. In return, the trees and other host plants provide them with energy in the form of sugars synthesized from atmospheric carbon. As well as improving nutrition and increasing the efficiency with which the water available in the soil is absorbed, trees also benefit from protection against root diseases[97]. Nutrients are not the only benefit that the host can receive. As ectomycorrhizas show tolerance to high concentrations of heavy metals, *P. arhizus* has shown potential for use in the bioremediation of contaminated soils[98].

Aside the vital role in the forest ecosystems, the genus *Pisolithus* offer a significant potential for application in biotechnology products. The main example is the use of *Pisolithus* pigments as natural dyes, as favorable alternative to synthetic ones[99,100].

1.2.2. Bioactive potential

It is estimated that around 25 to 30% of all drugs recognized as therapeutic agents are derived from natural products[101,102].

Studies have shown the bioactivity of compounds present in fungi used for medicinal purposes, such as phenolic compounds, antioxidants (gallic acid, protocatechuic acid, ellagic acid), among others[103].

Based on a series of studies on the interaction between *P. tinctorius* and its host plants, Marx (1977)[95] highlighted the presence of allelochemicals in this fungus. It was found that this fungus releases substances with the potential to affect the development of other organisms[95].

As mentioned earlier, this fungus is known for its ability to establish symbiotic relationships with host plants, boosting their growth in a more vigorous and healthy way, even in situations where the environmental conditions are not the most suitable for the plant in question[97,104].

The main potential of *P. arhizus* lies in its economic value due to its ability to boost reforestation programs. This fungus–host interaction raises the concentration of certain compounds, such as terpenes, in the host, whose function is to protect it against fungal infections[105].

The *Pisolithus* genus has been proven to have the ability not only to establish a barrier and neutralize factors such as low soil fertility, high temperatures and inadequate soil pH, but also to enhance the absorption of nutrients such as magnesium, phosphorus, sulphur, potassium, calcium, arsenic, cadmium, cobalt, copper, lithium, molybdenum, nickel and vanadium[105,106].

Many plants and animals depend on symbioses with fungi for resource acquisition and defense. For example, *Laccaria bicolor* has been shown to produce phytohormones, such as the auxin indole-3-acetic acid (IAA), which modify the morphology of the roots of its host plants[107]. Wagner et al. (2016)[108] found that the fungal-derived secondary metabolite, D'orenone, can promote the branching of hyphae of ectomycorrhizal *fungus Tricholoma vaccinum* and the production and release of IAA, the phytohormone responsible for cell differentiation and growth in plants. Similarly, D'orenone also had stimulating effects on lateral root length and root cortex cell hypertrophy in seedlings of the host tree *Picea abies*[108].

Concerning *Pisolithus,* several studies point to its potential as a source of pharmacological natural compounds. Main properties include the anticancer, antimicrobial, antioxidant and immunosuppressant potential.

In the context of the production of compounds with anti-cancer properties, it was identified that the triterpene pisosterol (Figure 5) , first isolated from the basidiocarps of *P. tinctorius* by Gill et al.[109], exhibits antitumor activity against various human cancer cell lines[110–114] (Table 1).

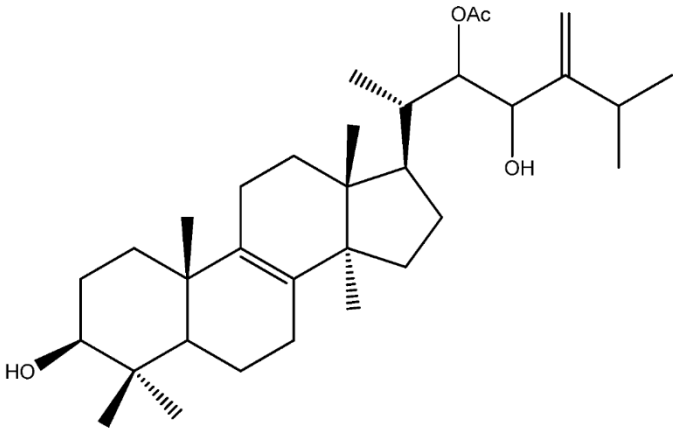

Figure 5. Chemical structure of pisosterol.

Montenegro et al. (2004)[110] tested the compound *in vitro* on mouse erythrocytes to infer about the effects on membrane rupture, developing sea urchin embryos, and on tumor cell lines, including CEM (human leukemia), HL-60 (human leukemia), B16 (murine melanoma), HCT-8 (human colon cancer), MCF-7 (human breast cancer), PC-3 (human prostate cancer) and SF-268 (human neuroblastoma), to assess cytotoxicity. The results showed no activity on erythrocytes and the development of sea urchin embryos. However, a notable inhibition of growth was observed in all tumor cell lines, especially in CEM, HL-60 and B16 cells.

Years later Montenegro et al. (2007)[111] carried out a study to see if pisosterol had the ability to induce cell differentiation. To do this, they used the HL-60 leukemia cell line as a cancer cell model and peripheral blood mononuclear cells (PBMCs) as representatives of non-cancerous cells. Inhibition of proliferation and induction of apoptosis by pisosterol was observed in the HL-60 leukemia cell line, with no evidence of cytotoxicity for normal cell lines, indicating selectivity of this compound for cancer cells.

Montenegro et al. (2008)[112] conducted a study aimed at an *in vivo* evaluation. Sarcoma 180 tumor cells were transplanted subcutaneously into female Swiss mice and treated with pisosterol for 7 days. Inhibition of tumor growth was described. To assess the toxicological

impact of pisosterol, morphological analyses were carried out. The treatment had an impact on the liver, showing hyperplasia of Kupffer cells, focal infiltration of inflammatory cells and centrilobular venous congestion, indicating that the liver is a target organ for pisosterol. However, the authors concluded that the damage may be reversible, since no signs of stromal fibrosis were detected, and the connective tissue was preserved.

Pereira et al.[113] described the impact of pisosterol on the glioblastoma multiforme (GBM) cell lines U343 and AHOL1. Treatment with pisosterol did not cause changes in the cell morphology of the two cell lines, indicating that this compound does not induce cell differentiation in these glioblastoma multiforme (GBM) cells. The cells were also treated to investigate abnormalities involving chromosome 8 or 8q24, where the C-MYC gene is mapped. The results indicated the absence of new chromosomal abnormalities after treatment, suggesting that pisosterol has no clastogenic and/or aneugenic effect.

The therapeutic potential of pisosterol in the treatment of cancer was also discussed by Ferreira et al.[114] using glioma cell lines. The cell viability and proliferation of U343, AHOL1, U 87MG and 1321N1 cells were notably reduced, with the inhibition of cell proliferation being attributed to the interruption of the G2/M phase and cell death by apoptosis.

Subsequently, Alves et al. (2015)[115] investigated the anticancer potential of compounds derived from *P. arhizus*, this time using extracts from the spores and testing their activity against human osteocarcinoma MG63, human breast carcinoma T47D, human colon adenocarcinoma RKO and normal human cerebral microvascular endothelial hCMEC/D3 cell lines. The results showed selective cytotoxicity for the cancer cell lines, encouraging further studies which, once again, indicated anticancer activity in some of the fractions obtained from the crude extract[116]. Simultaneous studies carried out on the same cell lines, but using biomass collected from the mycelium, also showed promising results in relation to the anticancer potential of *P. arhizus* extracts[117].

More recently, Parisi et al.[118] reported the isolation and elucidation of the structure of thirteen new triterpenoids, in addition to two already known, from extracts of *P. arhizus* basidiocarps. Analysis of the set of isolated compounds revealed that 24-methylanost-8,24(31)-diene-3 β ,22 ε -diol and 24(31)-epoxylanost-8-ene-3 β ,22S-diol showed induction of moderate cytotoxicity in a dose-dependent manner in the U-87MG and Jurkat cancer cell lines, without showing cytotoxicity in the HaCaT normal keratinocyte cell line.

Table 1. Compounds and extracts of *Pisolithus arhizus* with anticancer activity.

Compound/Extract	Biological material	Mechanism/Effect	Assays	Reference
Pisosterol	Basidiocarp	Antitumor	MTT: CEM (human leukemia) and HL-60 (human leukemia), B16 (murine melanoma), HCT-8 (human colon cancer), MCF-7 (human breast cancer), PC-3 (human prostate cancer) and SF-268 (human neuroblastoma) cell lines	[110]
Pisosterol	Basidiocarp	Antitumor	MTT: -Trypan blue exclusion -NBT reducing activity -Naphthyl acetate esterase activity -Antiproliferative effect (inhibition of DNA synthesis) HL-60 human promyelocytic leukemia cells; Peripheral blood mononuclear cells (PBMC)	[111] [119]
Pisosterol	Basidiocarp	Antitumor	Mice bearing Sarcoma 180 tumor cells	[112]
Pisosterol	Basidiocarp	Antitumor	Mitotic index GBM (glioblastoma multiforme) cell lines (U343 and AHOL1) Fluorescence *in situ* hybridization analysis with a locus-specific probe for C-MYC	[99] [120]
Pisosterol	Basidiocarp	Antitumor	MTT: Trypan blue exclusion; Flow cytometry; Staining with Annexin V-FITC/PI qPCR and western blotting U343, AHOL1, U-87 MG 1321N1 cell lines	[114]

DCM/MeOH and EtOAc/MeOH crude extracts	Spores	Antitumor	MTT: human osteocarcinoma cell line MG63, human breast carcinoma cell line T47D, human colon adenocarcinoma cell line RKO, and normal human brain capillary endothelial cell line hCMEC/D3	[115]
24 (31)-epoxylanost-8-ene-3β, 22S-diol.	Fruiting bodies	Cytotoxic activity on U87MG and Jurkat cell lines	MTT: U87MG (human glioblastoma), Jurkat (human T-lymphocyte), and HaCaT (human epidermal keratinocyte) cell lines	[118]
24-methyllanosta-8,24 (31)-diene-3β,22ε-diol	Fruiting bodies	Cytotoxic activity on U87MG and Jurkat cell lines Apoptotic effect	MTT: U87MG (human glioblastoma), Jurkat (human T-lymphocyte), and HaCaT (human epidermal keratinocyte) cell lines	[118]

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt

The pharmacological potential of this fungus has been documented in several studies, in particular its antimicrobial potential[121] (Table 2).

This fungus produces two antibiotic compounds, hydroxybenzoylformic acid (pisolitin A) and hydroxymandelic acid (pisolitin B) (Figure 6). They have been shown to inhibit spore germination and act in the lysis of hyphae of a significant number of phytopathogenic and dermatogenic fungi, which sheds light on how *P. arhizus* can protect its hosts against pathogenic microorganisms[122].

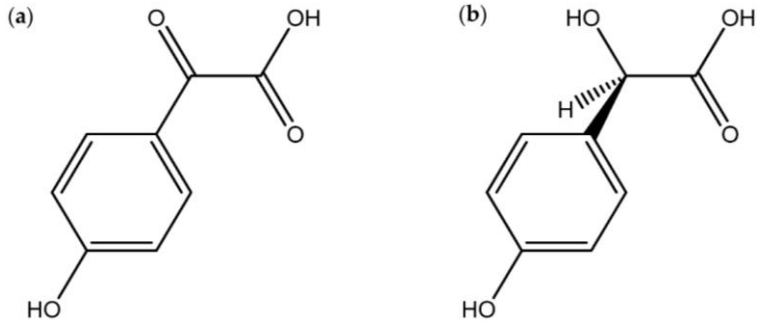

Figure 6. Chemical structures of pisolithin A (a) and pisolithin B (b).

These two acids also demonstrated a significant ability to inhibit the mycelial growth of various phytopathogenic fungi, such as *Rhizoctonia solani, Verticillium dahliae* and *Pyrenochaeta terrestris.* In addition, they showed an inhibitory effect on phytopathogenic oomycetes, such as *Pythium debaryanum,* as well as dermopathogenic fungi, including *Microsporum gypseum* and *Trichophyton equinum*. In certain situations, the inhibition was more pronounced when compared to other antifungal agents, such as nystatin and polyoxin D[122].

Carmo (2019)[123] isolated and identified four secondary metabolites from *P. tinctorius* extracts, subjecting them to separate tests to assess their antimicrobial activity. These compounds included a P56 ceramide, 5-hexadecenoic acid (Figure 7) and two triterpenes: pisolactone and 7,22-dien-3-ol, 24-methyl lanostane[123] (Figure 8).

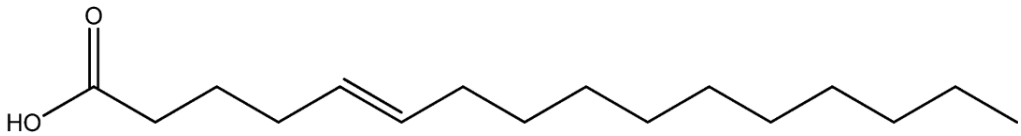

Figure 7. Chemical structure of 5-hexadecenoic acid.

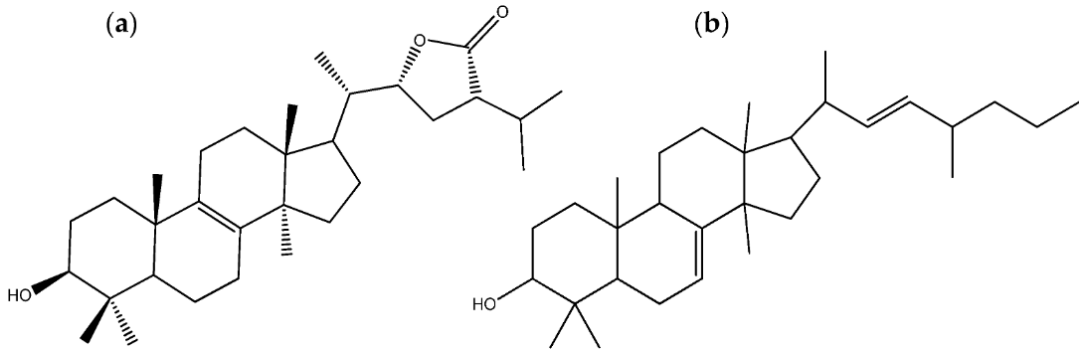

Figure 8. Chemical structures of the triterpenoids pisolactone (a) and 7,22-dien-3-ol, 24-methyl lanostane (b).

Vaidya et al. (2005)[124] reported the ability of *Pisolithus* sp. extracts to inhibit the growth of *Escherichia coli, Salmonella typhi, Kleibsiella pneumoniae* and *Pseudomonas aeruginosa* at a concentration of 10 mg/ml, and also a low inhibition of the growth of *Staphylococcus aureus*[124].

Table 2. Extracts of *Pisolithus arhizus* with antimicrobial activity.

Compound/Extract	Biological material	Mechanism/Effect	Assays	Reference
Pisolithin A **Pisolithin B**	Mycelium	Antifungal	Compared GI50 values with synthetic compounds (R)-mandelic acid, benzoylformic acid, and racemic p-hydroxymandelic acid Bioassay on spore germination inhibition	[122]
Pisolactone	Basidiocarp	Antimicrobial Antimycobacterial	Broth microdilution method Gram-positive: *Enterococcus* spp.*; Staphylococcus aureus; Bacillus cereus* Gram-negative: *Shigella sonnei; S.flexneri* Fungi: *Cryptococcus gattii; Cryptococcus neoformans*	[123]
7,22-dien-3-ol, 24 methyl lanostane **hexadec-5-enoic acid** **Phytoceramide**	Basidiocarp	Antibacterial	Broth microdilution method Gram-positive: *S. aureus, B. subtilis* Gram-negative: *Enterobacter aerogenes, Shigella sonnei, Morganella morganii, Shigella flexneri, Pseudomonas aeruginosa, Klebsiella pneumoniae*	[123]
Ethyl acetate extracts	Basidiocarp Spores	Antimicrobial	Agar well diffusion Activity against phytopathogenic fungi	[125]
Ethyl acetate extracts	Mycelium filtrate	Antimicrobial	Mycelium growth in solid medium Activity against phytopathogenic fungi	[126]
Hydroethanolic extracts	Basidiocarp	Antimicrobial	Broth microdilution Activity against multidrug-resistant Gram-negative and Gram-positive bacteria	[127]

Antioxidant[127–133] and immunosuppressants compounds[134,135] were also identified (Table 3).

In 2011, Reis et al. conducted a comparative analysis of the tocopherol composition and antioxidant properties of the fungus, both *in vivo* and *in vitro.* In this study, fruiting bodies and mycelia were used to assess the respective antioxidant capacities, revealing a remarkable antioxidant efficacy associated with *P. tinctorius,* especially when the corresponding mycelium structure was used[128].

The high antioxidant activity of *P. arhizus* compared to other macrofungi (*Amylosporus guaraniticus, Gloeophyllum striatum, Hydnopolyporus fimbriatus, Inonotus splitgerberi, Inonotus rickii, Lentinus lindquistii, Laccaria fraterna, Trametes cubensis*) was also demonstrated in the study by Campi[129].

Similarly, Pringle showed that the ethanolic and aqueous extracts of *P. tinctorius* had the highest antioxidant activity of the four fungi tested (*Pisolithus tinctorius*, *Russula capensis*, *Imleria badia, Pleurotus ostreatus*)[132].

More recently, Martins (2023) evaluated the antioxidant activity of hydroethanolic extracts from eight macrofungi (*Butyriboletus regius, Ganoderma lucidum, Inonotus hispidus, Lanmaoa fragrans, Pisolithus tinctorius, Suillellus luridus, Suillellus mendax, Xerocomus subtomentosus*). *P. tinctorius* stood out as one of the fungi with the highest antioxidant activity values, assessed by three different methods: 2,2-di(4-tertoctylphenyl)-1-picrylhydrazyl (DPPH), 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and ferric reducing antioxidant power (FRAP)[127].

Table 3. Compounds and extracts of *Pisolithus arhizus* with antioxidant and immunosuppressive activity.

Compound/Extract	Biological material	Mechanism/Effect	Assays	Reference
Tocopherols Methanolic	Mycellium Basidiocarp	Antioxidant	DPPH radical-scavenging activity; -Reducing power -Inhibition of b-carotene bleaching.	[128]
Ethanolic	Basidiocarp	Antioxidant	DPPH radical absorbance	[129]
Methanolic Ethanolic	Basidiocarp	Antioxidant	DPPH radical scavenging activity Metal chelating power	[130]
Ethanolic	Basidiocarp	Antioxidant	Rel Assay Kits Total antioxidant status (TAS) Total oxidant status (TOS) Oxidative stress index (OSI)	[131]
Ethanolic Aqueous	Basidiocarp	Antioxidant	DPPH radical scavenging activity FRAP	[132]
Hydroethanolic	Basidiocarp	Antioxidant	DPPH radical scavenging activity ABTS radical scavenging activity FRAP	[127]
Hydroethanolic Methanolic	Basidiocarp	Antioxidant	DPPH radical absorbance	[133]
Pisolactone	Basidiocarp	Immunosuppressive	Proliferation of mouse spleen lymphocytes stimulation with concanavalin A Lipopolysaccharide	[134]
Lanosterol	Basidiocarp	Immunosuppressive	Chromatin immunoprecitpitation assay	[135]

DPPH: 2,2-di(4-tertoctylphenyl)-1-picrylhydrazyl

ABTS: 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

FRAP: ferric reducing antioxidant power

2. Aim of the dissertation

All data gathered from previous works supports the potential of *P. arhizus* to be used as a source of compounds with interesting bioactivity properties.

Following the results obtained by Alves (2017)[116], Fernandes (2017)[117] and Lima (2023)[136], the objective of this dissertation was to continue investigating the cytotoxicity of the extracts originating from the *P. arhizus* mycelium and culture medium against both cancer and normal cell lines, in order to obtain further conclusions about which fractions possess higher activity.

Considering this main goal, the following specific tasks were defined:

- to prepare mycelium and culture medium crude extracts and fractionation;
- to assess the cytotoxicity of extracts and fractions against cancer cell lines;
- to perform a bioassay-guided isolation approach to purify secondary metabolites.

This thesis aims to trigger further research into the bioactive compounds of *Pisolithus* for innovative applications in addressing health challenges.

3. Materials and Methods

3.1. *Pisolithus arhizus*

The mycelium biomass and mycelium growth medium used were previously obtained by Lima, (2023)[136].

Specimens of *P. arhizus* were collected from a forest in northern Portugal and packed in paper bags. In a laboratory environment, the surface of the specimen was sanitized with a 70% ethanol solution and dried with absorbent paper to remove impurities and environmental residues, as well as possible spores that had already dispersed.

Small fragments were cut with a sterile scalpel and transferred to Petri dishes containing Potato Dextrose Agar (PDA) (Figure 9).

Figure 9. Isolated mycelium on PDA medium. Retrieved from Lima, (2023)[136].

After wrapping the plates with Parafilm, they were incubated at 25 ºC and monitored daily for contamination. After the growth period, the plates were removed from incubation. The mycelium was cut into small square pieces and placed in sterile plastic vials, following the same procedure with the surrounding PDA medium (Figure 10).

Figure 10. Plastic cups with the mycelium collected. Retrieved from Lima, (2023)[136].

The vials were frozen at –20 ºC and subjected to freeze-drying.

3.2. Extraction

The extraction protocol was previously optimized by Alves, (2017)[116] and Fernandes, (2017)[117]. First, a crude extract was obtained with a solution of dichloromethane–methanol in a ratio of 2 to 1 [DCM:MeOH (2:1)]. The dried biomass was placed in a vat and covered with the extraction solution, using a total of 2.39 L for the mycelium and 690 mL for the culture medium. The extraction process was repeated several times, recycling the extraction mixture. The vat was then left to rest to allow extraction to take place.

The biomass was filtered with the extraction solution into a pre-weighed round-bottomed flask. The extraction solvents were removed by evaporation under low pressures using a rotary evaporator (BUCHI R-210 Rotary Evaporator). The process was carried out at a temperature of 30 °C in the water bath and -8 °C in the condenser. The atmospheric pressure was gradually reduced until it reached 70 mbar, taking care to avoid boiling and consequent loss of sample. The final dry crude extract was stored at -20 ºC.

The extraction process is represented in Figure 11.

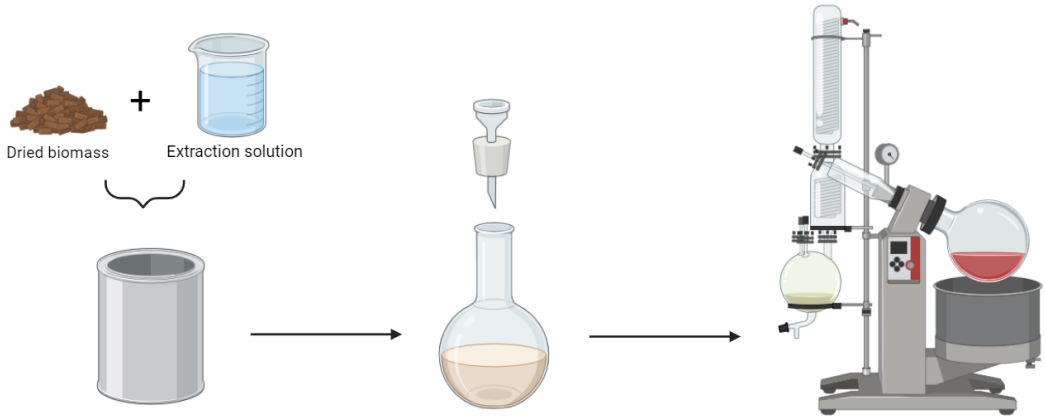

Figure 11. Schematic diagram of the extraction process. Created in BioRender.com.

In total, 4.9 g of mycelium and 1.8 g of culture medium were extracted.

3.3. Fractionation

The fractionation of crude extracts was carried out by Vacuum Liquid Chromatography (VLC), using a system composed of a vacuum pump, a Buchner funnel, an adapter and a round-bottom collection flask (Figure 12).

Figure 12. Vacuum Liquid Chromatography system.

The 250g of silica gel (stationary phase) were packed into the column with vacuum support. Initially, a mixture of hexane/ethyl acetate (Hex/EtOAc 9:1) was used as an eluent to saturate the column. This procedure was repeated until the silica was completely compacted. A Whatman filter paper was placed over the silica, always keeping a small amount of eluent mixture to keep the silica solvated. The crude extract was suspended in Hex:EtOAc (9:1) and transferred to the top of the column using a Pasteur pipette. A series of different mixtures with increasing polarity were then loaded onto the column, resulting in nine different fractions, ranging from A, with the lowest polarity, to I, with the highest polarity. The fractions were collected in round-bottomed flasks (Figure 13) and all the solvents were dried under reduced pressure in a rotary evaporator.

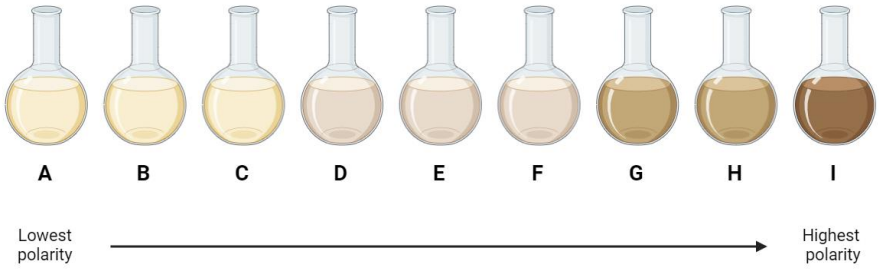

Figure 13. Nine fractions, ranging from A (lowest polarity) to I (highest polarity). Created in BioRender.com.

The eluent mixtures and their volumes are presented in Table 4.

Table 4. Eluents for the Vacuum Liquid Chromatography fractionation procedure of the medium and mycelium.

Fraction	Eluent mixtures	Volume medium (mL)	Volume mycelium (mL)
A	90% Hex 10% EtOAc	150	250
B	70% Hex 30% EtOAc	150	250
C	60% Hex 40% EtOAc	150	250
D	50% Hex 50% EtOAc	150	250
E	30% Hex 70% EtOAc	150	250
F	20% Hex 80% EtOAc	150	250
G	100% EtOAc	150	250
H	75% EtOAc 25% MeOH	150	250
I	100% MeOH	500	1250

Hex: Hexane; EtOAc: Ethyl acetate; MeOH: Methanol

The dried fractions were dissolved in hexane and ethyl acetate and transferred to pre-weighed 40 mL flasks. They were evaporated again to determine the weight of the dry mass.

3.3.1. Sub-Fractionation of fraction C of the mycelium

The fraction with higher anticancer activity (fraction C of the mycelium) was submitted to sub-fractionation, using automatic flash chromatographer (BUCHI C-850 FlashPrep).

All the mass of fraction C of the mycelium were sub-fractionated in a prepacked cartridge. Here it was used 25 g of silica gel (Silicycle) as stationary phase and a variety of solvents mixtures with increasing polarity as mobile phase, as hexane ethyl acetate and methanol.

3.4. High-Performance Liquid Chromatography (HPLC)

The results from the cytotoxic essay revealed a strong cytotoxic activity in the fractions 2 and 3 from sub-fractionation of fraction C of the mycelium.

Given this, High-Performance Liquid Chromatography (HPLC) was used to verify the chromatography profile of this set of fractions, evaluating its complexity and establishing the number of main components. HPLC fractionation is a process used for the separation and purification of complex mixtures, based on their chemical properties and interactions with the stationary phase and mobile phase, as they pass through the column.

The HPLC was performed using a Waters 1525 Binary HPLC Pump, with a normal phase Luna 5u Silica 100A 250 x 10 mm column, with an isocratic mixture of 80% Hex and 20% EtOAc and a 3.0 ml/min flow, with a dual slit UV/Vis absorbance detector set at 280 and 254 nm.

3.5. Cytotoxicity assay

3.5.1. Cell cultures

Cytotoxicity tests using the fractions obtained from both the mycelium and the culture medium of *P. arhizus* were carried out against cancer cell lines: colon adenocarcinoma cell line (RKO) and human neuroblastoma cell line (SH-SY5Y). Cytotoxicity was also assessed using the normal fibroblast cell line 3T3-L1. The source and densities for each cell line are described in Table 5.

Table 5. Cell lines used in the cytotoxicity assays, origin and recommended cell density.

Cell line	3T3-L1	RKO	SH-SY5Y
Cell type	Fibroblast	Colon adenocarcinoma	Neuroblastoma
Image			
Origin	American Type Culture Collection (ATCC)	American Type Culture Collection (ATCC)	American Type Culture Collection (ATCC)
Cell density (cell/mL)	3.3×10^4	3.6×10^4	1.5×10^5

The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco), pH 7.4, supplemented with 10% fetal bovine serum, 2.5 μg/ml fungizone and penicillin-streptomycin (100 IU/ml and 10 mg/ml, respectively).

The cells were incubated at 37 ºC in a humidified atmosphere with 5% CO_2 and the culture medium was renewed every 2 days.

3.5.2. Cell passages

When the cells reached 80-90% confluence, a cell passage was performed.

The first step consisted of removing the previous medium and washing the cells with 2 mL of warm phosphate buffered saline (PBS) (Gibco). Next, 1 mL of TrypLE Express enzyme (1x) (Gibco) was added to separate the cells from the flask wall and incubated for 3-5 min. After incubation, 4 mL of medium were added to inactivate the enzyme. The cell suspension was then transferred to a Falcon tube and centrifuged for 5 min at 1200 rpm. The supernatant was removed, and the sediment was resuspended in 1 mL of fresh medium. 50-100 μL of cell suspension were transferred to new culture flasks containing medium (25 cm^2 with 4 mL or 75 cm^2 with 10 mL) and incubated as described above.

3.5.3. Cell counting

To perform the cell count, 20 μL of the cell suspension were mixed with 20 μL of trypan blue. The cell concentration was determined using a Neubauer chamber and Trypan Blue dye, which interacts with cells that have a damaged membrane. Viable cells remained colorless, while dead cells were stained blue.

3.5.4. Cell viability assay

To test the cytotoxicity of each fraction against all the cell lines, the fractions were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 1 mg/mL. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out to study the effect of the extracts on cell viability.

The 3T3-L1, RKO and SH-SY5Y cell lines were seeded in 96-well culture plates at densities described in Table 5, and incubated for 24 h to ensure cell adhesion. After adhesion, the culture medium was removed, and the cells were exposed to the extracts in new medium for an

incubation time of 24 and 48 h. Each treatment was carried out in quadruplicates, except for the negative control, which had 8 replicates.

A schematic of the 96-well plate is shown in Figure 14.

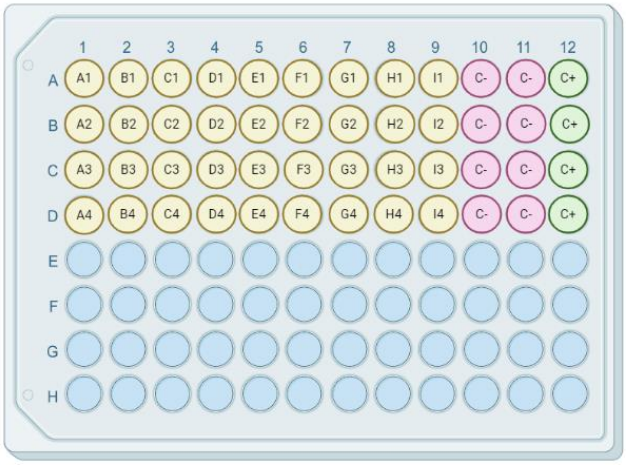

Figure 14. 96 well plate scheme for the cell viability assays. Created in BioRender.com.

After 24 and 48 h of exposure, 20 µL of MTT at 1 mg/mL were added to each well and incubated for 3 h. After incubation, the medium was removed and replaced with 100 µL of DMSO, since formazan crystals are only soluble in organic solvents.

The optical density (OD) was read at 562 nm using the Biochrom Ltd. microplate reader, UK EZ Read 800 Plus running Galapagos Expert software.

The positive control consisted in 20% DMSO. The negative control consisted in 1% DMSO. Cytotoxicity was expressed as a percentage of cell viability, considering 100% viability in the solvent negative control.

3.5.5. Dose response assay

From the viability assays using 3T3-L1 and RKO cells, fractions B and C were found to be toxic. In order to confirm this cytotoxicity, a dose response assay was performed to evaluate the effect of different concentrations of fractions.

Fractions B and C were dissolved in DMSO and diluted to the final concentrations of 10, 7.5, 5, 2.5, 1.25, and 0.625 µg/mL. 3T3-L1 and RKO cells were seeded on 96 well plates following the same method as described above. The positive and negative controls were also the same as described before.

The OD was measured as previously described and viability calculated using the same procedure as in the previous section.

3.6. Statistical Analysis

Microsoft Office Excel 2016 was used to organize the data from MTT assay. The results are presented as means ± standard deviation (SD) from three independent experiments, when compared with control results.

Normality of data from the cell viability assay was assessed through the Shapiro-Wilk test. Statistical analysis was performed using the statistical analysis of simple variance – ANOVA, with a significance defined as $p < 0.05$. One-Sample T-Test was used to determine significant differences between the treatments and the negative control (test value set at 100%).

All statistical analyses were performed in IBM SPPS Statistics 28.0. Graphs were made using GraphPad Prism 8.0.2 for Windows.

4. Results

4.1. Extraction and fractionation results

The final extraction was carried out using DCM–MeOH (2:1) as the extraction solution.

In total, 2390 mL of solvent were used to extract 4.9 g of mycelium, and 690 mL of solvent to extract 1.8 g of culture medium, summarized in Table 6.

Table 6. Volume of solvent used and dried extracts recovered after the extraction process.

	Volume of solvent used (mL)	Amount of dried extract recovered (g)
Mycelium	2390	4.9
Medium	690	1.8

After transferring the mixtures from the round-bottomed flasks to pre-weighed 40 mL flasks and evaporating the solvents in a rotary evaporator under reduced pressure, the fractions had the weights shown in Table 7.

Table 7. Weight of the fractions resulted from the fractionation of the medium and mycelium crude extracts.

Fraction	Eluent mixture	Dried fraction medium (mg)	Dried fraction mycelium (mg)
A	90% Hex 10% EtOAc	6.2	18.8
B	70% Hex 30% EtOAc	1.3	71.5
C	60% Hex 40% EtOAc	4.0	205.5
D	50% Hex 50% EtOAc	2.7	36.6
E	30% Hex 70% EtOAc	6.3	8.7
F	20% Hex 80% EtOAc	0.3	4.6
G	100% EtOAc	0.5	79.6
H	75% EtOAc 25% MeOH	7.5	4051.3
I	100% MeOH	1686.6	832

Hex: Hexane; EtOAc: Ethyl acetate; MeOH: Methanol

4.2.Sub-fractionation results

For the sub-fractionation of fraction C of the mycelium, a total of 65 test tubes were collected. The reorganization of the test tubes, according to the obtained chromatogram, outcome in 5 fractions. This involved transferring the substances into pre-weighed vials, subjecting it to evaporation under reduced pressure conditions, and subsequently determining the mass that was recovered, as detailed in Table 8.

Table 8. Fraction obtained with each tube and mass recovered from fraction C of the mycelium.

Fractions	Tubes	Mass (mg)
C_1	2-6	8
C_2	7-21	109.5
C_3	22-38	49
C_4	39-42	9.5
C_5	43-65	0.6

The fractionation process was performed with 205.5 mg of fraction C of the mycelium. After evaporating the solvents, the weight of the dried fractions was 176.6 mg, which corresponds to an recovery percentage of 85.94%.

4.3.High-Performance Liquid Chromatography (HPLC)

Fractions 2 and 3 from sub-fractionation of fraction C of the mycelium were combined because they were the fractions that showed the most promising results. The chromatographic profile shown one major component and the aim was to isolate this substance.

The following figure shows the general chromatography profile obtained through HPLC, and the following divisions in fractions (Figure 15).

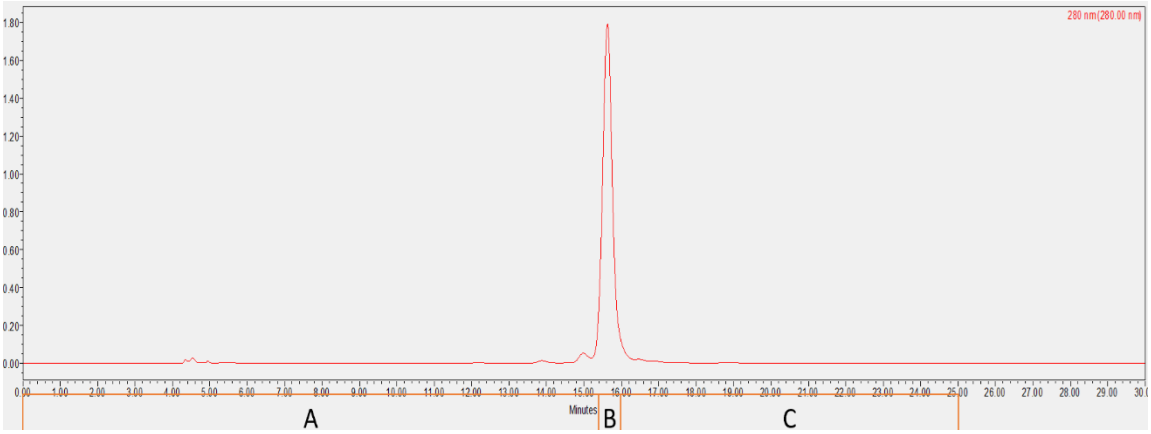

Figure 15. Chromatographic profile of the fraction 2+3 from sub-fractionation of fraction C of the mycelium.

After obtaining three different fractions and transferring them from the flasks to previously weighted vials and the solvents were evaporated on a rotatory evaporator with reduced pressure, the sub-fractions showed the following weights, presented in Table 9.

Table 9. Weight of the extract from subfractions of the fraction 2+3 from sub-fractionation of fraction C of the mycelium.

Fractions	Mass (mg)
A	91.6
B	10.4
C	5.7

4.4. Cytotoxic activity of the fractions

All fractions and sub-fractions were evaluated for cytotoxicity against 3T3-L1, RKO and SH-SY5Y cell lines, using the MTT assay. The initial fractions A-I (Table 7) were tested for cell viability at a concentration of 10 µg/ml with time of exposure of 24 and 48 h.

The fraction was considered cytotoxic when the percentage of cell viability was lower than 70%. To evaluate the effectiveness of the solvent and the measurement system, a positive control of 20% DMSO was used. This concentration was known to kill the cells and served as a benchmark for comparison. For the negative control, 1% DMSO was used, and its value represented the untreated cells thus determining the 100% of cell viability.

Results of the One-Sample T-test are represented by the symbols above the bars; *P<0.05; **P<0.01; ***P<0.001.

4.4.1. 3T3-L1 cell line

The following figures show the results of the cytotoxicity test carried out for the fractions A-I on 3T3-L1 cells (Figures 16-21).

Regarding the effect of the *P. arhizus* culture medium on the 3T3-L1 cell line (Figure 16), it was found that none of the fractions displayed cytotoxic activity.

However, some fractions did induce an increase in cell viability at 24 h, with emphasis on fractions A and I, with the values of 124.49 and 123.46%, respectively.

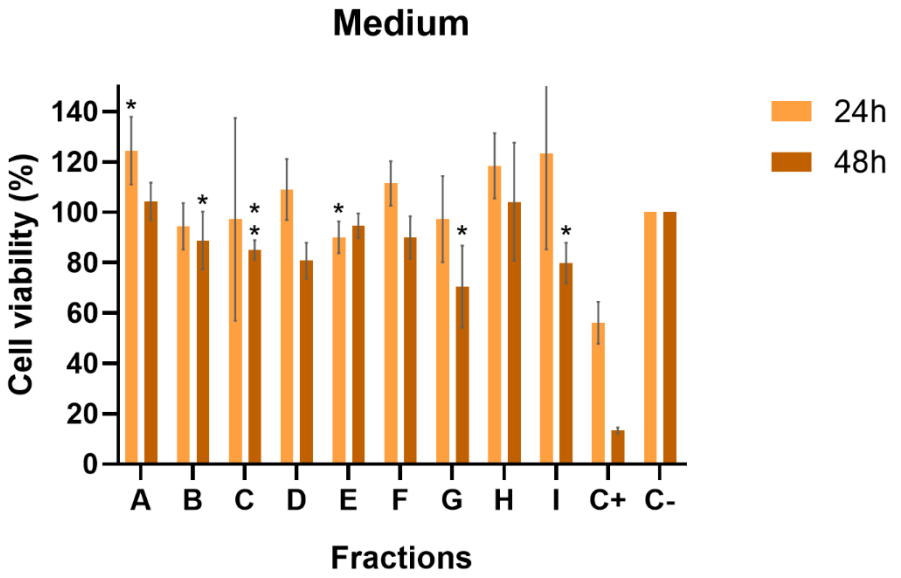

Figure 16. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the 3T3-L1 cell line on medium, at timepoints t=24 h and t=48 h.

Regarding the effect of the *P. arhizus* mycelium on the 3T3-L1 cell line (Figure 17), a percentage of cellular viability lower than 70% was registered after an incubation period of 24 h in fractions B and C, with values of 70 and 51.25%, respectively, and after 48 h of incubation in fractions B and C, with values of 56.50 and 27.31%, respectively.

Percentages of cell viability above 120% were shown after a 48 h period of incubation in fractions A and G, with the values of 137.31 and 120.30%, respectively.

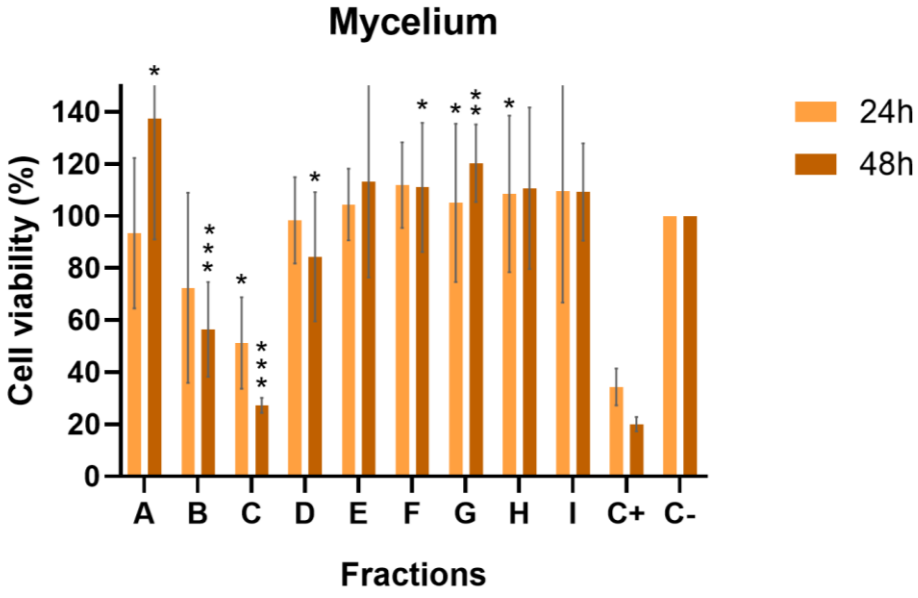

Figure 17. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the 3T3-L1 cell line on mycelium, at timepoints t=24 h and t=48 h.

Regarding the effect of different concentration of the fraction B of the mycelium (Figure 18), the results showed that none of the fractions displayed cytotoxic activity.

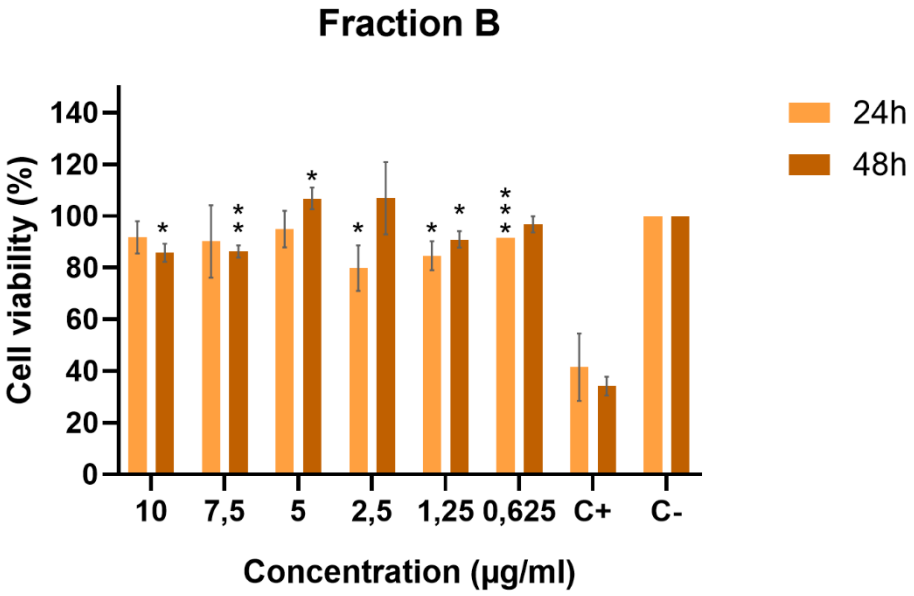

Figure 18. Effect of each concentration on the viability of the 3T3-L1 cell line on fraction B of the mycelium, at timepoints t=24 h and t=48 h.

Regarding the effect of different concentrations of the fraction C of the mycelium (Figure 19), a percentage of cellular viability lower than 70% was registered after an incubation period of 48 h in concentrations of 10 and 7.5 µg/mL, with cell viabilities of 70 and 65.44%, respectively.

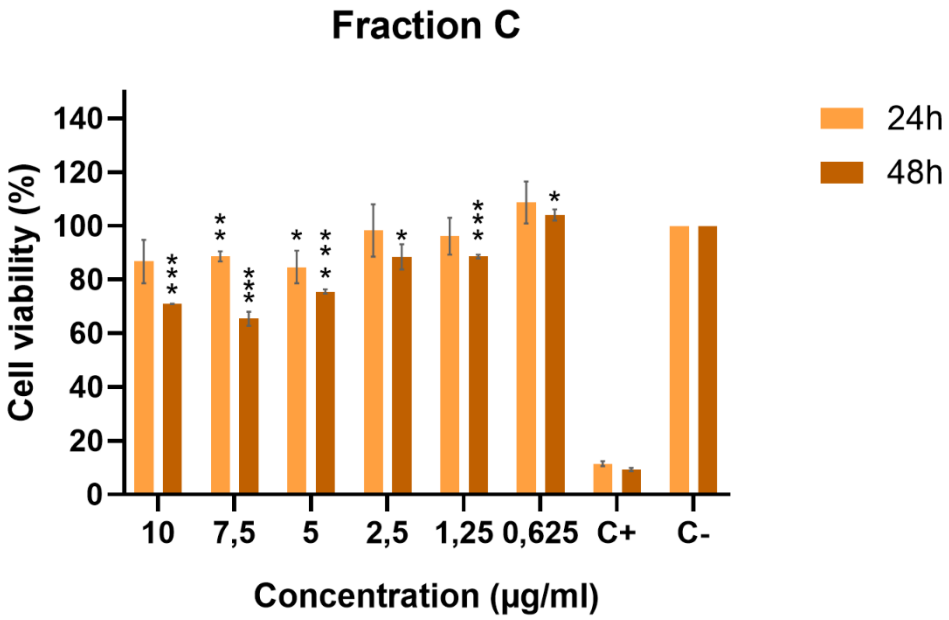

Figure 19. Effect of each concentration on the viability of the 3T3-L1 cell line on fraction C of the mycelium, at timepoints t=24 h and t=48 h.

Regarding the effect of sub-fractions of fraction C of the mycelium (Figure 20), an incubation period of 48 h showed a cellular viability percentage below 70% in fractions 2 and 3, with values of 65.87 and 41.89%, respectively.

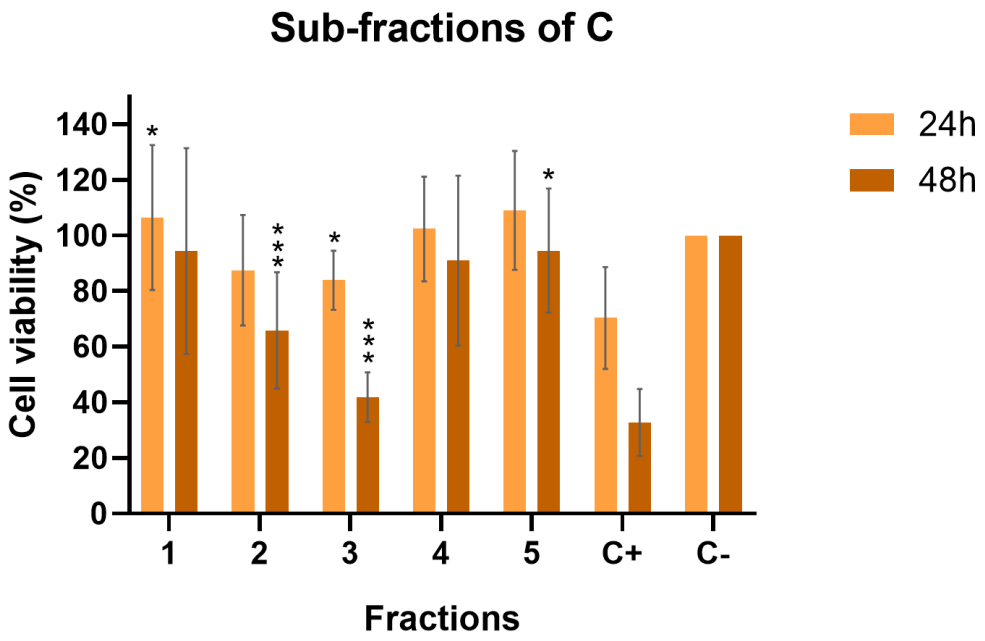

Figure 20. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the 3T3-L1 cell line on sub-fractions of fraction C of the mycelium, at timepoints t=24 h and t=48 h.

Regarding the effect of sub-fractions 2+3 of fraction C of the mycelium (Figure 21), it was found that none of the fractions displayed cytotoxic activity.

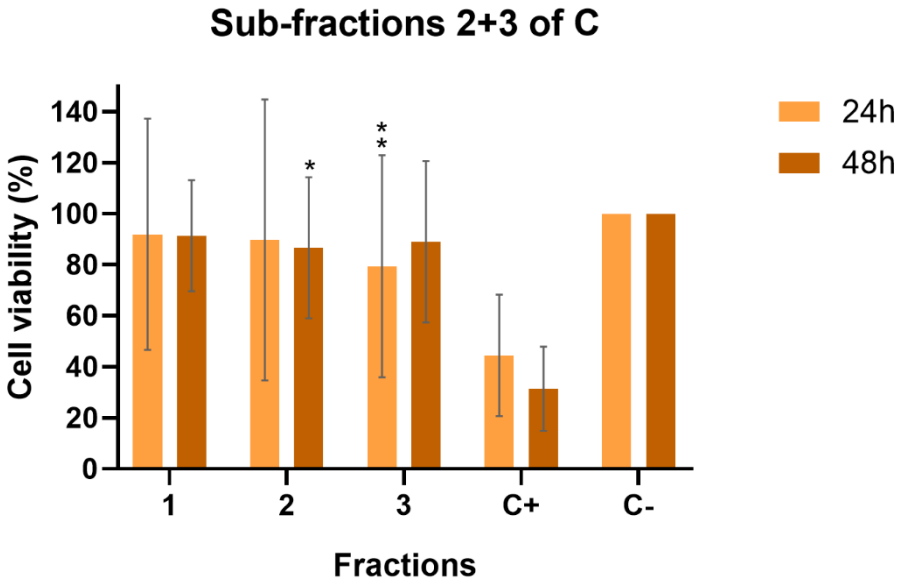

Figure 21. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the 3T3-L1 cell line on sub-fractions 2+3 of fraction C of the mycelium, at timepoints t=24 h and t=48 h.

4.4.2. RKO cell line

The following figures show the results of the cytotoxicity test carried out for the fractions A–I on RKO cells (Figures 22–27).

Regarding the effect of the *P. arhizus* culture medium on the RKO cell line (Figure 22), a percentage of cellular viability lower than 70% was registered after an incubation period of 24 h in fractions A and D, with values of 59.51 and 64.02%, respectively.

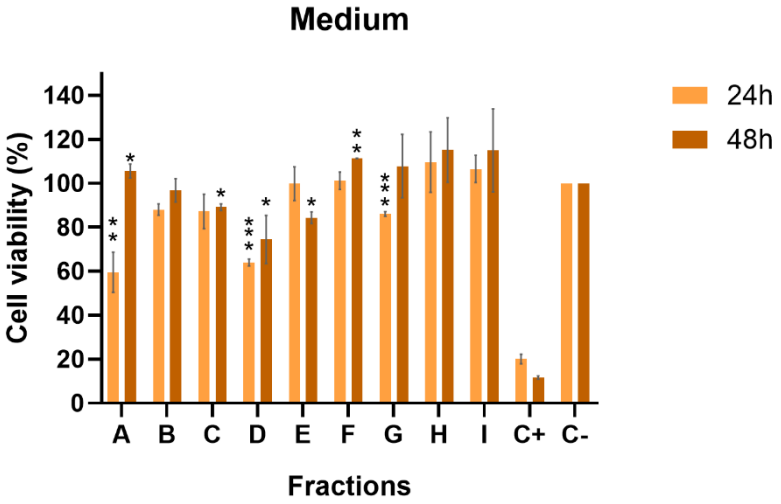

Figure 22. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the RKO cell line on medium, at timepoints t=24 h and t=48 h.

Regarding the effect of the *P. arhizus* mycelium on the RKO cell line (Figure 23), a percentage of cellular viability lower than 70% was registered after an incubation period of 24 h in fractions B and C, with values of 48.54 and 32.97%, respectively, and after 48 h of incubation in fractions B and C, with values of 37.48 and 24%, respectively.

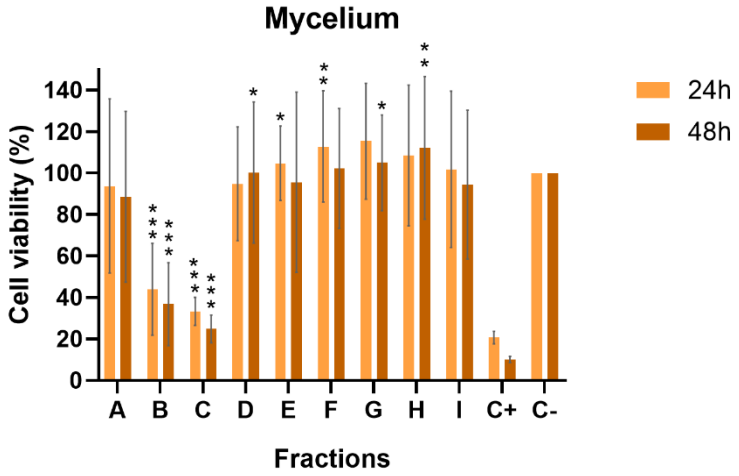

Figure 23. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the RKO cell line on mycelium, at timepoints t=24 h and t=48 h.

Regarding the effect of different concentration of the fraction B of the mycelium (Figure 24), all concentrations were found to be not toxic, with cell viability higher than 70%.

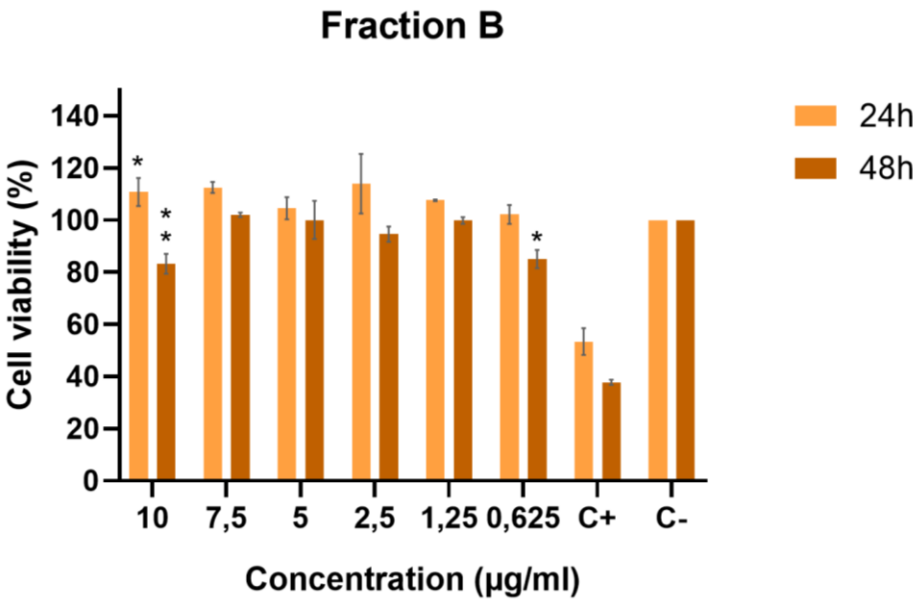

Figure 24. Effect of each concentration on the viability of the RKO cell line on fraction B of the mycelium, at timepoints t=24 h and t=48 h.

Regarding the effect of different concentration of the fraction C of the mycelium (Figure 25), a percentage of cellular viability lower than 70% was registered after an incubation period of 24 h in concentrations of 10, 7.5 and 5 µg/mL, with values of 47, 54.77 and 65.96%, respectively, and after 48 h of incubation in concentrations of 10, 7.5 and 5 µg/mL, with values of 25.86, 37.37 and 45.40%, respectively.

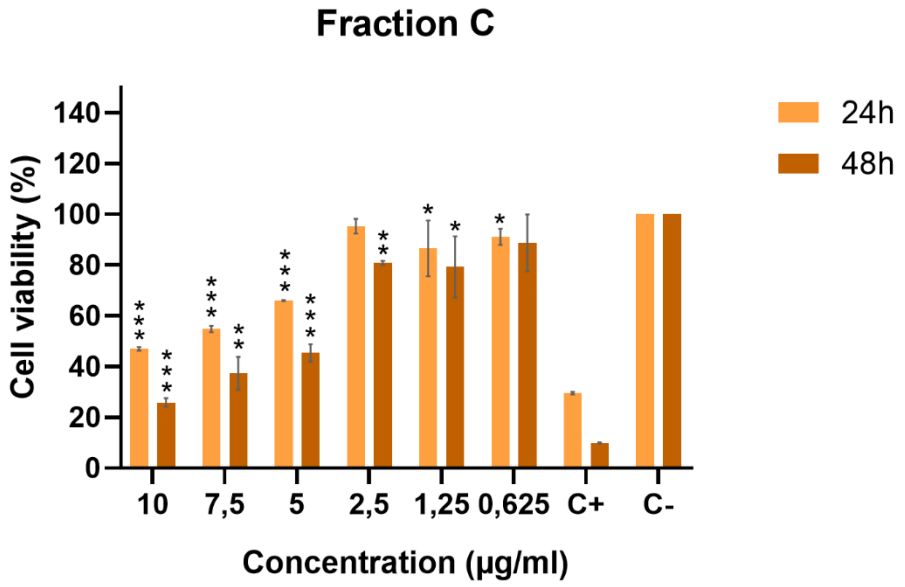

Figure 25. Effect of each concentration on the viability of the RKO cell line on fraction C of the mycelium, at timepoints t=24 h and t=48 h.

Regarding the effect of sub-fractions of fraction C of the mycelium (Figure 26), a percentage of cellular viability lower than 70% was registered after an incubation period of 48 h in fractions 2 and 3, with values of 63.84 and 60.86% respectively.

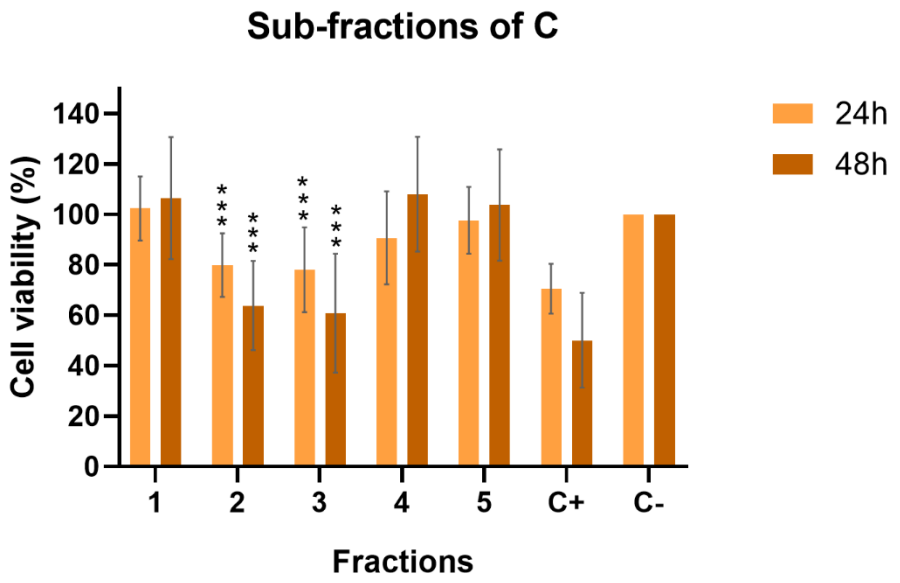

Figure 26. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the RKO cell line on sub-fractions of fraction C of the mycelium, at timepoints t=24 h and t=48 h.

Regarding the effect of sub-fractions 2+3 of fraction C of the mycelium (Figure 27), a percentage of cellular viability lower than 70% was registered after an incubation period of 24 h in fraction 1, with a value of 66.52%.

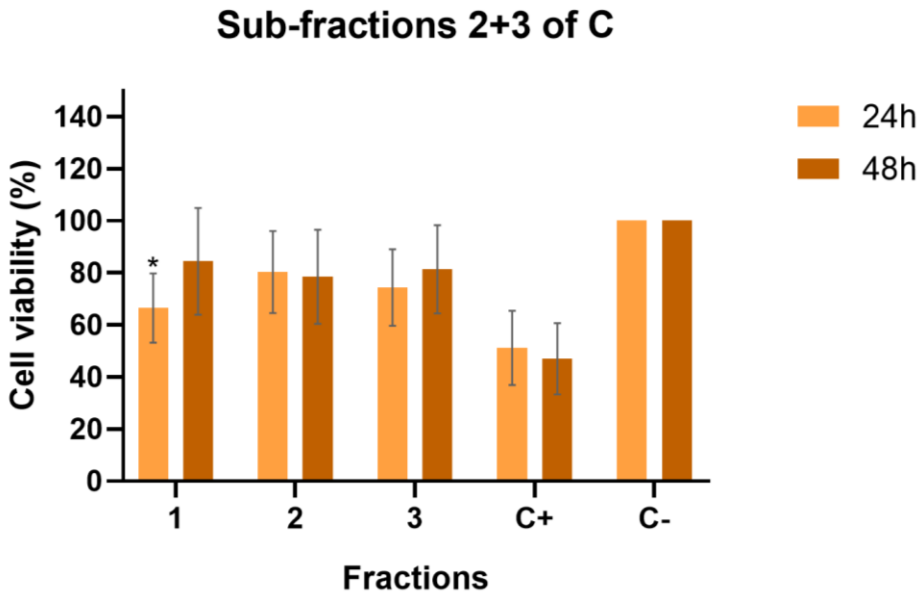

Figure 27. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the RKO cell line on sub-fractions 2+3 of fraction C of the mycelium, at timepoints t=24 h and t=48 h.

4.4.3. SHSY5Y cell line

Regarding the effect of sub-fractions 2+3 of fraction C of the mycelium (Figure 28), a percentage of cellular viability lower than 70% was registered after an incubation period of 24 h in fractions 1 and 2, with values of 60.86 and 68.21%, respectively, and after 48 h of incubation in fraction 1, with a value of 54.99%.

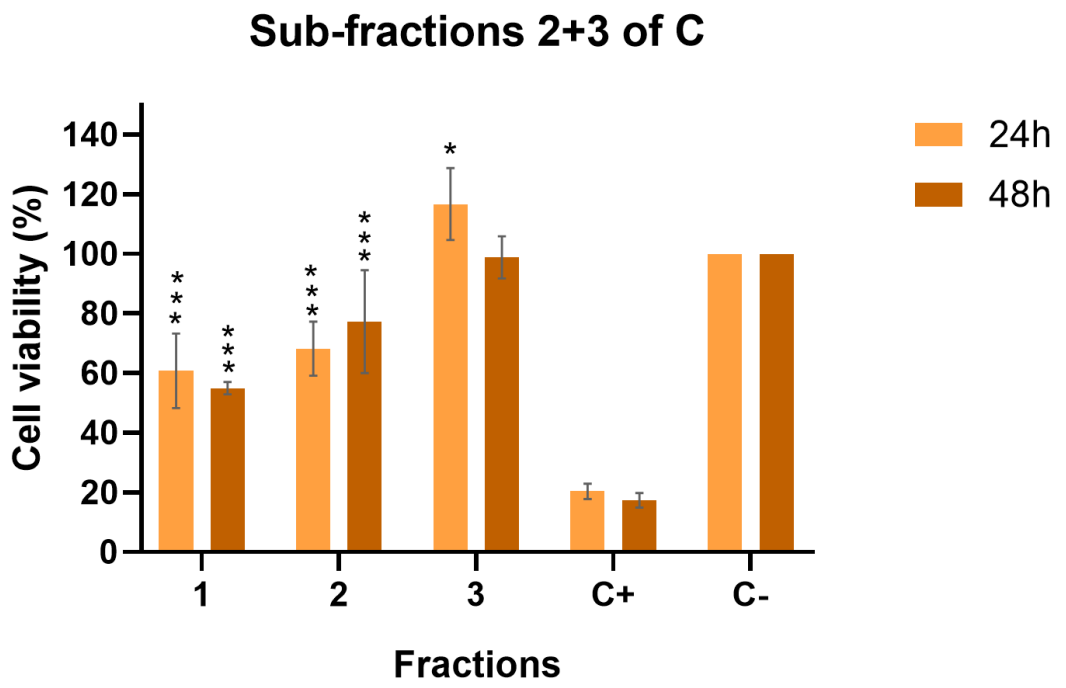

Figure 28. Effect of each fraction, at a concentration of 10 µg/mL, on the viability of the SHSY5Y cell line on sub-fractions 2+3 of fraction C of the mycelium, at timepoints t=24 h and t=48 h.

5. Discussion

Humanity is constantly searching for new substances that can improve biological functions and thus make people fitter and healthier. Macrofungi in general are vital to modern medicine and represent an endless source of compounds that are modulators of tumor cell growth[137].

In this work the anticancer potential of DCM/MeOH extracts obtained from *P. arhizus* mycelium and culture medium was evaluated against the RHO, SH-SY5Y and 3T3-L1 cell lines. The solvent mixture of DCM/MeOH in a 2:1 ratio for the extraction process was selected based on findings from prior studies[115,117]. This solvent mixture was found to be most effective for extracting compounds from both *P. arhizus* mycelium and culture medium.

The fractionation process was conducted using VLC, a method known for its efficiency in separating complex natural product mixtures. The solvents were selected based on protocols established by Alves et al. (2015)[115] and Fernandes (2017)[117], enabling effective separation of compounds in the crude extract according to their polarity. The most non-polar compounds were eluted first, followed by those with increasing polarity.

The masses obtained in the different fractions and after drying in the rotatory evaporator system, revealed large differences, from a minimum weight of 0.3 mg for the medium and 4.6 mg for the mycelium (both fractions F) to a maximum weight of 1686.6 mg for the medium (fraction I) and 4051.3 mg for the mycelium (fraction H). The substantially higher mass of the final fractions can be explained by the addition of MeOH in the last steps of fractionation. This solvent can dissolve some silica in the mobile phase, incorporating it into the fractions. Additionally, this suggests that most of the extracted compounds have a higher affinity for polar solvents.

The HPLC technique allowed sub-fractionation of fractions 2 and 3 from the sub-fractionation of fraction C of the mycelium in three new fractions (Figure 15), with the goal of separating different compounds and obtaining pure substances. Given the very strong anticancer activity observed in fraction B, it would be beneficial to test its purification through sub-fractionation in future studies.

The fractions obtained from the HPLC fractionation were submitted for LC-MS analysis to dereplicate the compounds by comparing them with known substances. However, due to time constraints, we were unable to obtain these results.

For the evaluation of the anticancer potential of fractions, the colon adenocarcinoma cell line RKO was selected. This selection was based in previous results by Alves et al. (2015)[115] and Lima (2023)[136].

Alves et al. (2015)[115] studies evaluated the anticancer potential of a DCM/MeOH and EtOAc/MeOH crude extracts from *P. tinctorius* spores. In this work the anticancer potential was evaluated against a battery of cell lines, including the human osteosarcoma cell line MG63, the human breast carcinoma cell line T47D, the human colon adenocarcinoma cell line RKO, and the normal human brain capillary endothelial cell line hCMEC/D3. As in the present work the cytotoxicity was evaluated by MTT assay. The MTT assay results revealed a significant reduction in cancer cell viability, with decreases of 92% and 88% for the DCM/MeOH and EtOAc/MeOH extracts, respectively, while the viability of normal cells was not significantly affected. The DCM/MeOH extract showed the most pronounced effects, reducing cellular viability to 12% in RKO and MG63 cells and 6% in T47D cells after 48 h of exposure. These findings highlight the broader anticancer potential of *Pisolithus*, extending beyond the basidiocarp to other structures of the fungal genus.

In 2023 a preliminary project focused on the anticancer potential of *P. arhizus* mycelium was conducted[136]. In this project, RKO and hCMEC/D3 cell lines were used. Cell viability was measured for each fraction on two time points (24 and 48 h). As for the hCMEC/D3 cell line, fraction B showed the greatest reduction in cell viability both at 24 and 48 h, indicating a more pronounced cytotoxic effect compared to other fractions. Fraction A initially induced moderate cytotoxicity at 24 h, but at 48 h an increase in cell viability occurred. All other fractions (C, D, E, F, G, H, I) did not show any cytotoxicity. As for the RKO cell line, similarly, fraction B stood out for significantly reducing cell viability in both time periods. All fractions, except fractions H and I, revealed cytotoxicity at 24 h. Most fractions stayed cytotoxic beyond this timepoint, except for fractions F and G, which managed to recover the viability. This means that most of the fractions are capable of inducing cytotoxicity in RKO cells, indicating the presence of compounds with potential anticancer activity in them. For this cell line, fraction B was selected for a dose response assay. This assay's results confirmed a decrease in cell viability with an increased in the fraction concentration. Positive and negative controls were consistent with expectations, validating the effectiveness of the test. The difference in response between the two cell lines indicates that the compounds may have specific targets or mechanisms of action that vary between different cell types.

However, the lack of data on the compounds responsible for the cytotoxicity makes additional studies essential and thus the present project follows these results.

For cytotoxicity evaluation the MTT assay was performed. This assay determines cell viability, indirectly indicating cell proliferation. This *in vitro* cell viability assay is particularly beneficial because it is easy, quick, cost-effective, and do not involve the use of animals[138]. This widely used colorimetric assay measures cell viability by assessing mitochondrial function. MTT is a water-soluble yellow tetrazole salt that is reduced by NAD(P)H-dependent oxidoreductase enzymes in metabolically active cells to form purple formazan crystals. These crystals can then be quantified using visible light spectrophotometry[138]. The reduction of MTT into formazan depends on the cell's metabolic rate. Therefore, slowly dividing cells will show slower MTT reduction rates, while cells with a high metabolism will reduce MTT more quickly[139].

In the present work, the cytotoxicity observed in RKO cell lines with some fractions, with minimal to no cytotoxicity towards 3T3-L1 cells, aligns with the findings of Alves et al. (2015)[115], where also demonstrated selective activity against cancer cell lines. This selectivity was observed with fraction C in the dose response assay.
Selectivity is desired when discussing a treatment for cancer, in which a selective action is expected with the lowest toxicity possible for the normal cells.

Regarding the results of the culture medium, in the 3T3-L1 cell line, no fraction showed cytotoxicity, with fractions A and I promoting cell growth within 24 h, with 124.49 and 123.46% (Figure 16), respectively. In the RKO cell line, only fractions A and D showed cytotoxicity of 59.51 and 64.02% (Figure 22), respectively, at 24 h.
Also on the RKO cell line, fraction B of the mycelium revealed cytotoxicity with cell viability dropping to 48.54% after 24 h and further to 37.48% after 48 h (Figure 23); Fraction C exhibited a stronger reduction in cell viability of only 32.97% after 24 h and 24% after 48 h (Figure 23). This reduction in cell viability indicates that these fractions contain compounds that can interfere with cell proliferation, which is a desirable characteristic in anticancer agents. From the initial fractions A-I, results revealed a high cytotoxicity of the fractions B and C of the mycelium. Of these fractions, fraction C deserved special mention, since it presented a very high and regular cytotoxicity. This pronounced cytotoxic effect may be due to the presence of

more potent or higher concentrations of active compounds within fraction C compared to fraction B.

Although toxicity was observed to fractions B and C of the mycelium against the cancer cell line, the same result was observed with the normal cell line 3T3-L1 although to a lesser extent. In fact, for the 3T3-L1 cell line fraction B exhibited a reduction in cell viability to 70% at 24 h and to 56.5% after 48 h (Figure 17). Fraction C showed even more pronounced cytotoxicity with cell viability dropping to 51.25% after 24 h and further to 27.31% after 48 h (Figure 17).

This suggests that fraction C contains potent bioactive compounds that warrant further investigation to determine their potential applications and underlying mechanisms of action.

The remarkable activity shown by fractions B and C prompted further studies, namely a dose response assay to determine the influence of the concentration of these fractions on cell viability of the cell lines. The highest concentration tested was the same concentration used for the cytotoxicity assays (10 µg/mL), with successive dilutions made for the remaining concentrations (7.5, 5, 2.5, 1.25 and 0.625 µg/mL).

For fraction B, in both cell lines, all concentrations were found to be not toxic (Figures 18 and 24). For fraction C, cell viability and concentration seem to have an inversely proportional relationship, where the lower the concentration of the fraction, the higher the cell viability, with more pronounced results for RKO cell line (Figures 19 and 25).

As fraction C had the results with higher cytotoxicity, we proceeded with its fractionation, in which we obtained five fractions. In both cell lines, the fractions 2 and 3 demonstrated cytotoxicity after 48 h, with values of 65.87 and 41.89% (Figure 20), respectively, for the 3T3-L1 cell line, and 63.84 and 60.86% (Figure 26), respectively, for the RKO cell line.

Fractions 2 and 3 from sub-fractionation of fraction C of the mycelium were combined because they were the fractions that showed the most promising results. HPLC of this mixture of fractions resulted in three new fractions. For the 3T3-L1 cell line, none of the fractions displayed cytotoxic activity (Figure 21). While in the RKO cell line, only fraction 1 demonstrated cytotoxicity after 24 h, with 66.52% (Figure 27).

From this we can conclude that fractions 2 and 3 of the sub-fractionation of fraction C of the mycelium, present cytotoxicity when tested individually, but do not present cytotoxicity when mixed, in the 3T3-L1 and RKO cell lines. These results lead us to hypothesize that there are compounds that when in combination exert a synergistic effect that reduces toxicity.

Considering some characteristics of the selected cell lines, RKO is a human colon adenocarcinoma cell line that is widely used in studies investigating the molecular pathways of cancer progression, such as cell cycle regulation, signal transduction, and metastasis. RKO cells are notable for their wild type p53 status, a rarity among cancer cell lines. This feature makes them particularly valuable for studying p53 functions and the cellular mechanisms of DNA repair and apoptosis in colorectal cancer[140]. Their rapid growth and high tumorigenicity make them a robust model for *in vivo* and *in vitro* cancer studies[141].

The 3T3-L1 cell line is an embryonic mouse fibroblast cell line. Fibroblasts are the primary cells of connective tissue and play a crucial role in providing structural support to various tissues. These cells are easy to culture and maintain, and provide a consistent and reproducible model system for various types of biological and biomedical research[142].

In order to test other cancer cell lines, the cytotoxic effects were also evaluated in the human neuroblastoma cell line SH-SY5Y. This cell line is frequently used in neuroscience and neurobiology research. These cells exhibit dopaminergic and adrenergic characteristics, making them a suitable model for studying neuronal function and neurotransmitter activity[143]. They are a valuable cell model for neurodegenerative disorders such as Parkinson's and Alzheimer's disease[144]. The results obtained with this cell line revealed that fraction 1 resulted in a significant decrease in viability, with 60.86 % at 24 h and 54.99% at 48 h, indicating a possible cytotoxic effect. Fraction 2 initially induced moderate cytotoxicity at 24 h, with 68.21%, but at 48 h an increase in cell viability occurred. Fraction 3 did not demonstrate any cytotoxicity. The observed results suggest that the tested extracts may have variable effects on neuronal health depending on the chemical composition of the fractions.

While RKO and SH-SY5Y cells are of human origin, 3T3-L1 cells are of mouse origin. Due to the unavailability of human fibroblasts, we chose to use the available 3T3-L1 cell line.

In general, comparing the impact of the cell viability in the cancer and normal cells, it was verified that they are both affected by the active compounds. Although, some fractions were shown to be more toxic to the cancer cells than to the normal ones.

It must be considered that (a) cell culture may not accurately replicate the *in vivo* conditions, potentially leading to interferences, and (b) a longer treatment duration and future assessments involving the co-culture of different cell lines may be necessary to better understand the cellular interactions and therapeutic responses.

Another explanation for the difference in cell viability between cancer and normal cells is their metabolic rate. Cancer cells are well known for having a higher metabolic rate, with increased glucose consumption to boost tumor proliferation, a phenomenon known as the Warburg effect[145]. Consequently, cancer cells can absorb more nutrients, including bioactive compounds, and thus exhibit toxic effects earlier and more acutely[146].

The significant cytotoxicity observed, particularly in fraction C, suggests the presence of potent secondary metabolites. Compounds such as polysaccharides, peptides, and phenolic compounds are known for their anticancer properties and may be present in these fractions. These compounds could be acting through various mechanisms, including induction of apoptosis in cancer cells; inhibition of cell proliferation; disruption of cellular metabolism; induction of oxidative stress leading to cell death. Compounds that could also be present include the well described pisosterol.

Following Gill et al.'s isolation of the pisosterol triterpene in 1989, numerous studies have focused on this compound[109].

These studies have demonstrated pisosterol's ability to inhibit tumor growth in seven different cell lines[110], reduce viable HL-60 cells without affecting normal PBMCs[111], and inhibit C-MYC gene amplification, thereby enhancing the ability to combat cancer cells[110,113].

Pereira et al. (2011)[113] also described the effect of pisosterol, but in the glioblastoma multiform (GBM) cell lines U343 and AHOL1. The treatment with three concentrations of pisosterol (0.5, 1.0, and 1.8 μg/mL) did not alter the cell morphology of the two cell lines, which is an indication that pisosterol does not induce cell differentiation in these GBM cells.

The potential of this triterpene in cancer therapy was also described on a panel of glioma cell lines by Ferreira et al. (2020)[114]. Cellular viability and proliferation of U343, AHOL1, U-87MG, and 1321N1 cells were significantly decreased in a dose-dependent manner (concentrations of 0.97, 1.94, and 3.50 μM), with inhibition of cell proliferation via the G2/M phase arrest and cell death by apoptosis.

Also, Montenegro et al. (2004)[110] and Burbano et al. (2009)[119] tested the activity of pisosterol, extracted from *P. tinctorius*, and compared it with chemotherapy drugs such as doxorubicin and etoposide. Pisosterol demonstrated only slightly lower potential. Therefore, it can be emphasized that the potential compounds present in the fractions demonstrating anticancer

activity could be used alongside conventional drugs. This combination could potentially reduce the side effects of these drugs, making the treatment less aggressive and more effective.

While the most relevant anticancer research on *Pisolithus* has focused on pisosterol, numerous other compounds have also been identified. Among these, various medium and long-chain saturated fatty acids, extracted from methanolic extracts of *P. tinctorius*, have demonstrated anticancer properties[105]. Notably, capric and lauric acids have been highlighted for their ability to induce apoptosis in colorectal, skin, and breast cancer cell lines[147–149].
Ergosterol peroxide, an ergosterol derivative isolated from the basidiocarps of *P. tinctorius, Microporus flabelliformis* and *Lenzites betulina,* has shown promising results against various cancer cell lines, including HT29 colon adenocarcinoma cells[134].
More recently, Parisi et al. (2023)[118] reported the isolation and structure elucidation of thirteen new and two previously known triterpenoids from chloroform and methanolic extracts of the basidiocarps of *P. arhizus.* Among the isolated compounds, 24-methyllanosta-8,24(31)-diene-3β,22ε-diol and the newly discovered 24(31)-epoxylanost-8-ene-3β,22S-diol demonstrated moderate, dose-dependent cytotoxicity against U-87MG and Jurkat cancer cell lines, while showing no cytotoxicity towards the normal keratinocyte cell line HaCaT.

The data obtained in the present work allowed us to confirm the presence of compounds with cytotoxic effects, validating the potential of this fractions to induce cell death in RKO cells. This reinforces the significance of further investigating these compounds for their medicinal applications. Cytotoxicity towards specific cell lines can offer therapeutic benefits, such as bioactive anticancer properties.

The observed toxicity in normal cells highlights the need for a detailed safety assessment of the compounds. Additional trials in animal models are essential to determine acute and chronic toxicity, as well as long-term effects.

A next step would involve the identification to compounds that are already known or compounds that we cannot find in databases or in the existing bibliography (potentially new substances). If a new substance is identified, it will be prioritized for isolation and subsequent structural elucidation.

The statistical analysis of data was performed using the Shapiro-Wilk test due to the small sample sizes, revealing a normal distribution of the results for all cell lines ($P > 0.05$). This allowed for the use of parametric testing to identify treatments where the group means deviated significantly from the negative control mean. The objective was to compare the means of each treatment group (characterized by the fraction and the corresponding time point) with the mean of the negative control group, which is always 100%. Therefore, the One Sample T-test was chosen, with the test value set to 100.

6. Conclusion

Fungi not only provides nutritious, protein-rich food, but some species also produce effective medicinal products. With their wide variety of species, they are a profitable means of both supplementing humanity's nutrition, and alleviating the suffering caused by certain types of illnesses[60].

Macrofungi can be an alternative source of new antimicrobial compounds, mainly secondary metabolites such as terpenes, steroids, anthraquinones, benzoic acid derivatives and quinolones, but also some primary metabolites such as oxalic acid, peptides and proteins[150].

The present thesis helped further the research into the untapped potential of the macrofungus *P. arhizus* as a source of bioactive compounds, and strengthen the results obtained in the past by Alves et al. (2015)[115], Fernandes (2017)[117] and Lima (2023)[136], regarding the anticancer properties of this species.

This study showed high cytotoxicity values of *P. arhizus* mycelium and culture medium extracts in different types of cancer cell lines, proving its potential as a possible future resource of anticancer compounds. In general, most of the fractions tested showed selective cytotoxicity towards the RKO cancer cell line, with little activity towards the 3T3-L1 normal cell line. The fraction with the highest activity was considerably more toxic to RKO cells than 3T3-L1 cells as well.

Although it was not possible to identify the compound responsible for this anticancer activity, it was possible to arrive at very simplified fractions, which allow rapid results to be obtained in future works. Thus, this study demonstrates that one or more compounds with highly elevated anticancer activity can be extracted from the purified mycelium and culture medium of this fungus. In this sense, fraction C is promising for the isolation of compounds with potential anticancer activity, since it presents selectivity to the cancer cells in relation to normal cells.

Furthermore, the isolation of a compound would possibly open the door for synthetic routes of production, eliminating the need to culture the fungus under laboratory conditions, and the discovery of analogues with more desirable properties than the original molecule. It will also be necessary to expand the assays to other cell lines, both normal and from other cancer subtypes, in order to determine if the compounds are truly selective for cancer cells, and if so, which types of cancer they can be applied to.

7. Future perspectives

Given the high cytotoxicity of fractions B and C, especially the consistent activity of fraction C, further investigations are warranted. Future research should focus on isolation and characterization (identifying and characterizing the specific bioactive compounds responsible for the observed cytotoxicity), mechanistic studies (understanding the underlying mechanisms of action of these compounds at the molecular level), *in vivo* studies (evaluating the efficacy and safety of these compounds in animal models to better replicate human physiological conditions) and synergistic studies (investigating the potential synergistic effects of combining these fractions or their active compounds with existing chemotherapeutic agents to enhance anticancer efficacy).

The MTT assay is a useful preliminary screening tool for bioactive compounds and for detecting direct impacts on cell metabolic activity. However, due to the limitations of the test, the toxicity of the bioactive compounds should be confirmed using other assays, such as the lactate dehydrogenase (LDH) test, which provides information about the integrity of cellular membranes[151,152].

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Appendices

Appendix I. Review Paper

Exploring the Bioactive Potential of *Pisolithus* (Basidiomycota): Comprehensive Insights into Antimicrobial, Anticancer, and Antioxidant Properties for Innovative Applications

Abstract

Addressing pressing health concerns, modern medical research seeks to identify new antimicrobials to combat drug resistance, novel molecules for cancer treatment, and antioxidants for inflammation-related diseases. *Pisolithus* (Basidiomycota) is a ubiquitous and widely distributed fungal genus in forest ecosystems, known for establishing ectomycorrhizal associations with a range of host plants, enhancing their growth, and conferring protection against biotic and abiotic stresses. Beyond ecological applications, *Pisolithus* yields bioactive compounds with medicinal potential. This comprehensive review explores the transversal biological activity of *Pisolithus* fungi, aiming to provide a thorough overview of their antimicrobial, anticancer, and antioxidant potential. The focus is on elucidating bioactive compounds within *Pisolithus* to trigger further research for innovative applications. Compounds from *Pisolithus* displayed antimicrobial activity against a broad spectrum of microorganisms, including antibiotic-resistant bacteria. The efficacy of *Pisolithus*-derived compounds matched established medications, emphasizing their therapeutic potential. In anticancer research, the triterpene pisosterol stood out with documented cytotoxicity against various cancer cell lines, showcasing promise for novel anticancer therapies. Pisolithus was also recognized as a potential source of antioxidants, with basidiocarps exhibiting high antioxidant activity. *In vivo* validation and comprehensive studies on a broader range of compounds, together with mechanistic insights into the mode of action of *Pisolithus*-derived compounds, are compelling areas for future research.

Keywords: biological activity; fungi; natural compounds; *Pisolithus arhizus; Pisolithus tinctorius*

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Appendix II. Poster presentation I

21852 | Evaluation of the anticancer potential of the macrofungus *Pisolithus arhizus* mycelium and culture medium

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Background & Aim: Macrofungi produce remarkable biomedical products that can make a significant contribution to health. *Pisolithus arhizus* is a fungal species of the phylum *Basidiomycota*. It is mainly found in association with the roots of some trees species, establishing a mutualistic relationship with them, facilitating the absorption of nutrients from the soil in exchange for carbohydrates produced by the host plant. As recently reviewed by us [1], this macrofungus has aroused great interest due to its promising therapeutic properties and bioactive effects. **Methods:** Crude extracts were prepared from the mycelium and culture medium of *P.arhizus* using a mixture of dichloromethane:methanol (2:1. The extracts were then separated into nine fractions using vacuum liquid chromatography. The fractions were tested for cytotoxicity against the RKO colon adenocarcinoma cell line and 3T3 fibroblasts cell line. Cell viability was assessed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay. **Results:** Preliminary results revealed a dose dependent cytotoxicity against RKO cell line with no significant cytotoxicity to 3T3 cell lines, in fractions B and C. Those fractions corresponded to extraction with 70% Hex:30% EtOAc and 60% Hex:40% EtOAc, respectively. **Conclusions:** The results indicate that *P.arhizus* mycelium is a source of compounds with anticancer activity. However, more in-depth studies are needed to analyze the chemical compounds from the various parts of *P. arhizus* and investigate their biological and toxicological activities. Exploring the bioactive potential of *P. arhizus* is key to developing a complete understanding of its therapeutic benefits.

Keywords: *Pisolithus Arhizus*, Macrofungi, Anticancer Potential.

740

Appendix III. Poster presentation II

POSTER 08

Evaluation of the anticancer potential of the macrofungus *Pisolithus arhizus* mycelium and culture medium

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Keywords: *Pisolithus arhizus*, macrofungi, anticancer potential.

References

[1] Oliveira RS, Preto M, Santos G, Silva AM, Vasconcelos V, Martins R. Exploring the Bioactive Potential of *Pisolithus* (Basidiomycota): Comprehensive Insights into Antimicrobial, Anticancer, and Antioxidant Properties for Innovative Applications. Microorganisms. 2024; 12(3):450. https://doi.org/10.3390/microorganisms12030450.