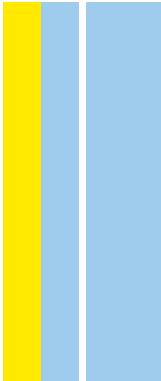


DISSERTAÇÃO DE MESTRADO
TOXICOLOGIA E CONTAMINAÇÃO AMBIENTAIS

Potential of the mycelium of the soil
fungus *Pisolithus tinctorius* as a source
of bioactive compounds

João Nuno Fernandes Fernandes



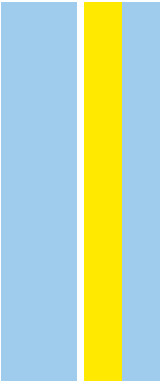
João Fernandes. Potential of the soil fungus *Pisolithus tinctorius*
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**POTENTIAL OF THE MYCELIUM OF THE SOIL FUNGUS *PISOLITHUS*
TINCTORIUS AS A SOURCE OF BIOACTIVE COMPOUNDS**

Dissertação de Candidatura ao grau de
Mestre em Toxicologia e Contaminação
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Universidade do Porto.

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ABSTRACT

Nowadays, cancer is one of the major causes of mortality in the world, and has been the focus of substantial interest for researchers of different areas. *Pisolithus*, an ectomycorrhizal fungus, has shown different biological potentials in previous studies, namely the anticancer and antimicrobial potential. In the present dissertation, the aim of study was the evaluation of the anticancer and antimicrobial potential of *P. tinctorius*, using specifically the isolated mycelium and fruiting body. Malt Extract Agar (MEA), Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) were tested in order to select the best nutrient medium to potency the mycelium growth. The influence of penicillin-streptomycin in the cultures was also tested to evaluate the influence in contamination rate. PDA showed to be the best nutrient medium, and also the cultures without penicillin-streptomycin showed the lowest contamination rate.

Extracts obtained from the lyophilized fruiting bodies and from the mycelium of *P. tinctorius*, using different solvents for the extraction (Dichloromethane-Methanol (2:1), Ethyl acetate-Methanol (2:1), Methanol and Dichloromethane), were assayed for cell cytotoxicity by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay on the colon adenocarcinoma cell line (RKO), human breast cancer cell line (T47D) and the human liver hepatocellular carcinoma cell line (HEPG2). Extracts obtained from the isolated mycelium using DCM-MeOH (2:1) showed higher activity on the cancer cell lines, comparing to the ones obtained from fruiting bodies and with different solvents. In order to infer about the cytotoxicity in human normal cells, cytotoxicity assays were also performed using normal human dermal micro vascular endothelial cell line (hCMEC/D3).

By a bioassay guided fractionation using the cancer cell lines, a Vacuum Liquid Chromatography (VLC) was performed in order to fractionate the crude extracts, obtaining nine fractions with different polarities. The results showed fractions with a very strong cytotoxic activity, with an accentuated reduction of the viability of the cancer cell lines, reducing this value to under 10% in the RKO, HEPG2 and T47D cell lines, after 72 h of incubation. ¹H Nuclear Magnetic Resonance analysis (NMR) was performed for the fractions that demonstrated highest cytotoxicity in cancer cell lines and highest viability on the normal human cell line, in order to

obtain information about the possible bioactive compounds present in each fraction. The fraction with more interest results was submitted to a High Pressure Liquid Chromatography (HPLC) to detect the possible compounds and also to obtain a sub-fractionation of the previous fraction.

The antimicrobial activity of each fraction obtained after the VLC fractionation was also tested using the agar disk-diffusion method, against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhimurium* and *Candida albicans*. This activity was also evaluated using the crude extracts of *P. tinctorius* mycelium and fruiting bodies, obtained with the 4 solvents tested. No antimicrobial activity was reported for none of the extracts or fractions tested.

Overall, the present work showed a strong anticancer activity of the extracts obtained from the *P. tinctorius* isolated mycelium in different types of cancer cell lines, proving its potential as a possible future resource of anticancer compounds.

RESUMO

O cancro é, hoje em dia, uma das maiores causas de mortalidade no mundo, e tem sido alvo de um interesse substancial por parte de investigadores de diferentes áreas. O *Pisolithus tinctorius*, um fungo ectomicorrízico, demonstrou já diversos potenciais biológicos em estudos anteriores, nomeadamente o seu potencial anticancerígeno e antimicrobiano. Na presente dissertação, o objetivo de estudo foi a avaliação do potencial anticancerígeno e antimicrobiano do *P. tinctorius*, utilizando especificamente o seu micélio isolado. O Malt Extract Agar (MEA), Sabouraud Dextrose Agar (SDA) e o Potato Dextrose Agar (PDA) foram testados com vista a selecionar qual dos três seria o melhor meio nutritivo para potenciar o crescimento micelial. A influência da penicilina-estreptomicina nas culturas foi também testada, avaliando a sua eficácia na taxa de contaminação. O PDA demonstrou ser o melhor meio nutritivo, e as culturas sem penicilina-estreptomicina demonstraram a taxa de contaminação mais baixa.

Os extratos obtidos através do corpo frutífero liofilizado e do micélio do *P. tinctorius*, utilizando diferentes solventes (DCM-MeOH (2:1), EtOAc-MeOH (2:1), MeOH e DCM) foram testados quanto à sua citotoxicidade, através de um teste de brometo 3-(4,5-dimetil-2-tiazolil)-2,5-difenil-2H-tetrazólio (MTT) em linhas celulares de adenocarcinoma do cólon (RKO), cancro da mama humano (T47D) e a linha celular de carcinoma hepatocelular do fígado humano (HEPG2). Os extratos obtidos a partir do micélio isolado utilizando DCM-MeOH (2:1) revelaram uma atividade superior em linhas de células cancerígenas, comparativamente aos obtidos a partir do corpo frutífero ou com diferentes solventes. Com vista a auferir acerca da citotoxicidade em linhas celulares humanas normais, os ensaios de citotoxicidade foram também efetuados utilizando uma linha normal de células endoteliais microvasculares dérmicas humanas (HCMEC/D3).

Efetuuou-se uma Cromatografia Líquida em Vácuo (VLC) com o objetivo de fracionar o extrato bruto, obtendo nove frações com diferentes polaridades, sendo que estas foram também submetidas a um ensaio MTT. Os resultados mostraram frações com uma atividade citotóxica bastante elevada, com uma redução acentuada da percentagem de viabilidade celular nas linhas de células cancerígenas, reduzindo este valor para um número inferior a 10% nas linhas RKO, HEPG2 e T47D, após 72 h de incubação. Procedeu-se também a análise por

Ressonância Magnética Nuclear ^1H (NMR) das frações que demonstraram maior citotoxicidade nas linhas de células cancerígenas e maior viabilidade na linha celular humana normal, com vista a obter informações acerca dos possíveis compostos presentes em cada fração e que são responsáveis pela bioatividade, sendo que a fração de maior interesse foi submetida a uma Cromatografia Líquida de Alta Eficiência (HPLC) para detetar os possíveis compostos presentes e também para obter um subfracionamento da fração utilizada.

A atividade antimicrobiana de cada fração obtida após o fracionamento por VLC foi também testada, utilizando o método de disco-difusão em agar, contra *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhimurium* e *Candida albicans*. Complementarmente, a atividade foi também avaliada utilizando os extratos brutos do micélio do *P. tinctorius* e do seu corpo frutífero, obtido a partir dos 4 solventes testados. Não foi reportada nenhuma atividade antimicrobiana de nenhum dos extratos testados.

De uma forma geral, o presente estudo demonstrou uma forte atividade anticancerígena dos extratos obtidos a partir do micélio isolado do *P. tinctorius* em diferentes linhas de células cancerígenas, provando o seu potencial como uma futura fonte de compostos anticancerígenos.

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ABREVIATIONS AND ACRONYMS

DCM – Dichloromethane

DCM-MeOH – Dichloromethane-Methanol

DMSO – Dimethyl Sulphoxide

DNA – Deoxyribonucleic Acid

DNA (RAPD) – Random Amplified Polymorphic DNA

ECM – Ectomycorrhizal

EtOAc-MeOH – Ethyl-acetate-Methanol

FBS – Fetal Bovine Serum

GBM – Glioblastoma Multiforme cell line

hCMEC/D3 – Normal Human Dermal Microvascular Endothelial cell line

HEPG2 – Human Liver Hepatocellular Carcinoma cell line

HL-60 – Human Promyelocytic Leukemia cell line

HPLC – High Performance Liquid Chromatography

HTS – High-throughput Screening

IC50 – Half Maximal Inhibitory Concentration

MEA – Malt Extract Agar

MeOH – Methanol

MG63 – Human Osteocarcinoma cell line

MH – Mueller-Hinton agar

MMN – Mannitol Nitrate Motility agar

MRSA – Methicilin-Resistant *Staphylococcus aureus*

MSSA – Methicillin-Sensitive *Staphylococcus aureus*

MTT – 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide reduction assay

NMR – Nuclear Magnetic Resonance

OD – Optical Density

PBMC – Peripheral Blood Mononuclear cells

PDA – Potato Dextrose Agar

RKO – Human Colon Adenocarcinoma cell line

SDA – Sabouraud Dextrose Agar

T47D – Human Breast Cancer cell line

VLC – Vacuum Liquid Chromatography

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1. General Introduction

Throughout history, different myths have been created about fungi. While the Egyptians believed that fungi were a gift from the Osiris God, the Romans thought that mushrooms were thrown into the earth through the rays sent by Jupiter. Greek soldiers found in the mushrooms an endless source of strength and courage, and in China mushrooms as food were considered the "elixir of life" (Chang & Miles, 1989). Fungi were also used in the Neolithic and Paleolithic eras (Samorini, 2011) and the oldest written record of mushrooms as a medicinal product is an Indian medical treatise, dated from 3000 BC (Islam, 2015)

Also throughout history, civilizations have developed a wide use and knowledge about fungi, distinguishing initially those most suitable for human consumption, in terms of nutritional value and flavour, and not suitable ones or causers of undesirable effects, from psychotropic reactions, to hallucinations or even death. The most consumed species were already referenced as having an excellent nutritional value, as well as a low content of lipids, proteins and carbohydrates, containing an essential range of amino acids (Mattila et al., 2000).

China is the pioneering country in the growth of medicinal mushrooms, responsible for about 70% of the production of this kind of therapeutic food, covering more than 60 species (Singh et al., 2011). About 50% of these species are exported to global market networks. In 1993, 1.95 million tons of mushrooms were produced worldwide – in 2003, the number rose to 3.19 million, a growth of 60% in only 10 years (FAOSTAT, 2004). Due to the high contact of some countries with fungi, as is the case in China, the development of this aspect leads to the fact that, in Eastern medicine, different species of fungi are used for a wide range of therapeutic purposes and medical treatments. In the 1960s, with the evolution of modern medical research, the extracts obtained from fungi began to show their effectiveness in several therapeutic contexts (Chang & Miles, 1989).

Agaricus, *Lentinula*, *Pleurotus*, *Auricularia*, *Volvariella* and *Flammulina* represent over 90% of the total world mushroom production (Borchers et al., 1999). Mushrooms in general have approximately 90% water by weight, and the remaining 10% are divided in 10% to 40% protein, 2% to 8% fat, 3% to 28%

carbohydrate, 3% to 32% fibre and 8% to 10% ash (Breene, 1990). Mineral content is mainly potassium, calcium, phosphorus, magnesium, iron, zinc and copper and most of the mushrooms contain also vitamins like niacin, thiamin, riboflavin, biotin and vitamin C, and a wide range of bioactive molecules like terpenoids, steroids, phenols and nucleotides (Breene, 1990; Miles & Chang, 2004; Mizuno et al., 1995).

The Fungi kingdom comprises a vast group of organisms that characteristically lack the presence of photosynthetic pigments, having a heterotrophic nutrition performed by absorption, which do not carry out carbon fixation and the nutrients are absorbed through the cell wall and the plasma membrane (Webster et al., 2007). In addition, they store glycogen as a reserve substance (Alexopoulos et al., 1996; Rogerson & Webster, 2007).

The current classification of fungi divides the same into seven main phyla, encompassing Ascomycota, Basidiomycota, Blastocladiomycota, Chytridiomycota, Glomeromycota, Microsporidia and Neocallimastigomycota, estimating the existence of 1.5 to 5.1 million species of fungi, of which only about 120,000 were officially described (Blackwell, 2011; Hibbett et al., 2007). Furthermore, the large percentage of existing species has not yet been studied, so it is necessary to exploit this vast majority for future research (Hawksworth, 2001).

The phylum Basidiomycota covers approximately 30,000 described species, being the equivalent to 20% of the total fungi described. They characteristically produce external sexed spores - the basidiospores - that vary according to the producing species (Guerrero & Homrich, 1983). These species are also often mycorrhizal formers, which have great importance in sectors such as agriculture and environmental aspects, since they have the capacity to increase the water absorption by the plants, facilitate the nutritive exchange and even facilitate the adaptation of vegetative species to the environment in which they are inserted (Miranda, 1993).

The mushrooms are historically considered a source of metabolites with bioactive potential (Papaspnyridi et al., 2011). Metabolites are essentially divided into primary and secondary metabolites. Primary metabolites correspond to essential elements for the functioning of the organism, being products of common routes

to all of them, such as sugars, amino acids and glycerol. The secondary metabolites are synthesised in the final stage of growth of the organism, near the stationary phase, covering a wide variety of compounds depending on the organism in question. Primary metabolites are essential for the growth, maintenance and reproduction of organisms, while the secondary metabolites confer some advantages to the producing organisms and are not considered essential. It is noteworthy that the synthesis of secondary metabolites is entirely related to the culture medium in which the organism is inserted, being this synthesis a response to external stimulation, such as changes in pH, temperature, among others (Madigan et al., 2004; Strohl, 2000).

Thorough studies carried out with fungi, as in the case of metabolites isolated from mushrooms, have long been associated with beneficial medical properties and have already been tested as antioxidants, anti-inflammatories, antivirals or even antitumor agents (Smith et al., 2002). Bahl (1983) reported that mushrooms can cure epilepsy, wounds, skin diseases, heart ailments, rheumatoid arthritis, fevers, diaphoretic, diarrhea, dysentery, cold, anesthesia, liver disease, gall bladder diseases and can be used as vermicides (Bahl, 1983). Jones & Lim (1990) reported that lentinan sulphate obtained from *Lentinus* species can inhibit HIV (Jones & Lim, 1990). Also, aqueous extracts from *Pleurotus sajor caju* proved to be beneficial in renal failure (Tam et al., 1986). Hobbs (2000) reported a wide range of medicinal uses of mushrooms, like cholesterol reduction, reduction of stress, memory improvement or asthma and allergy treatment. Different polysaccharides have been studied and tested regarding their biological potential level. For example, several bioactive compounds, obtained from *shiitake* (*Lentinula edodes*), were isolated and purified and their biological activity was reported, such as the antiviral and antitumor effects (Chihara et al., 1969; Sugui et al., 2003). In addition, mushrooms such as *Ganoderma lucium* (Reishi), *Lentinus edodes* (Shiitake), *Inonotus obliquus* (Chaga) among many others have been used for hundreds of years in countries such as Korea, China, Japan and Russia. A huge variety of scientific studies have been carried out in the fight against cancer, specifically of the stomach, prostate and lung (Hobbs, 2000; Stamets, 2000; Wasser & Weis, 1999; Ying et al., 1989). Polysaccharides obtained from *L. edodes* were boosted from successive efforts of several researchers, in such a way that in 1993 the isolated fungal polysaccharides already represented about 25% of the drugs used to help fight cancer in the Japanese market (Mizuno,

1999). Polysaccharides, isolated from several fungi, have been submitted to both *in vivo* and *in vitro* studies, and have shown very positive results regarding anticancer activity. In relation to *in vitro* tests, the toxicity of these compounds has been tested using 3- (4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay, which evaluates fundamentally the mitochondrial activity and viability of different cancer cell lines (Borchers et al., 1999); for *in vivo* tests using suckling rats, polysaccharides exhibited a stimulation of macrophages in the production of cytokines, which identify the target tumour cells to be eliminated by the cytotoxic T lymphocytes. This is a result that showed direct effect of fungal polysaccharides on the human immune system (Chan et al., 2009). The polysaccharide B-glucan is one of the metabolites with most important and transversal biological activities, since their mechanisms of action involve their recognition as non-self molecules, stimulating the immune system with their presence (Chen & Seviour, 2007; Patel & Goyal, 2012).

In addition to the anticancer activity of these compounds, the antimicrobial activity of compounds obtained from fungi is also receiving special attention. In addition to metabolites excreted extracellularly by the mushrooms, some molecules such as the polysaccharides, also mentioned above, present antimicrobial activity, and several metabolites have presented antibiotic properties. This fact makes it clear that mushrooms deserve consideration for their efficiency for medicinal purposes, and with the development and increase of bacterial resistance to commercial antibiotics, mushrooms can represent a great promise in the production of alternative medicines (Stamets, 2000; Wasser & Weis, 1999). The literature presents evidence on several taxonomic groups of fungi that have not yet been systematically explored in the search for secondary metabolites that may have antimicrobial effects (Gloer, 2007).

Analysing a global prism about mushrooms and their biological potentialities, the study of this same group of organisms deserves emphasis, in order to find compounds that may eventually be the source of future therapeutic and medicinal products.

1.1. *Pisolithus tinctorius* – Characteristics and Geographical Distribution

Pisolithus is a widely distributed fungal genus that belongs to the Sclerodermaceae family and Basidiomycota phylum, and is considered an ectomycorrhizal gasteromycete, associated with plants including members of Pinaceae, Myrtaceae, Fagaceae, Mimosaceae, Dipterocarpaceae and Cistaceae (Cairney, 2002; Chambers & Cairney, 1999; Marx, 1977). The presence of fungi from this genus has been reported in a wide range of habitats, being forests, orchards, urban sites and eroded and mine site soils some of them (Castellano & Trappe, 1991; Malloch & Kuja, 1979; Marx, 1977).

Different *Pisolithus* species differ in aspects like morphological and molecular characteristics, symbiotic efficiency, ecology, physiology and geographical distribution, being present in about 33 countries, distributed by six continents (Anderson et al., 2001; Burgess et al., 1995; Cairney, 2002; Lamhamedi et al., 1990).

Distinct *Pisolithus* isolates demonstrate strong variations in the morphology, mainly of the sporocarp and the basidiospore, although different species were recognised as *P. tinctorius* by Bronchart et al., 1975. Regarding the sporocarp structure, there is a considerable heterogeneity reported between the different *Pisolithus* species, and it is noteworthy that this genus was widely grouped as *P. tinctorius* or *P. arhizus* by Coker and Couch (1928) and Watling et al. (1995), being legitimate to consider this one of the main species of these fungi (Bronchart et al., 1975; Coker & Couch, 1928; Watling, 1995).

Different reports of the worldwide presence of *Pisolithus* were done in years, and an effort is being made to classify and characterise the huge variety of species under the genus. In 1988, Martin et al. reported that different species of this genus can be associated with different host plant species, obtaining internal transcribed spacer (ITS) sequence data in order to conclude about the isolates sequence similarity, using *Pisolithus* isolates from Kenya and Australia, obtained from species like *Azadirachta*, *Eucalyptus* and *Pinus* (Martin et al., 1988). Also, the same author analysed 102 isolates and reported the existence of at least 10 different species of *Pisolithus*, obtaining these isolates also from different geographical origins, like Kenya, Senegal, Malaysia, USA, Portugal, South Africa,

Japan, Thailand, Spain, Nicaragua, France, Wales, China, Brazil, India, Philippines and Morocco (Martin et al., 2002).

In 1995, Watling et al. described several species like *Pisolithus kisslingi* E. Fisch, *Pisolithus pusillum* Pat. and *Pisolithus aurantioscabrosus* Watling et al. based on basidiospore and sporocarp morphology, obtained from tropical South-East Asia (Watling, 1995). In the same year, Burgess et al. analysed different electrophoretic patterns of mycelial proteins obtained from different *Pisolithus* isolates collected from different geographical regions of Australia, and demonstrated that the geographical origin and basidiospore morphotypes can be correlated with the groupings based on similarities in polypeptide patterns (Burgess et al., 1995).

Later, in 1998 and 1999, through random amplified polymorphic DNA (RAPD) analyses, a considerable genetic variation was demonstrated within *Pisolithus* isolates from Australia, Brazil, Europe, North America, Scandinavia and the Philippines (Anderson et al., 1998; Junghans et al., 1998; Sims et al., 1999). Also, regarding the examination of restriction fragment length polymorphism (RFLP) patterns of internal transcribed spacer (ITS) and intergenic spacer (IGS) regions of the rDNA gene complex, it was showed that distinct polymorphism patterns could be seen in *Pisolithus* isolates from Australia, Kenya and Indonesia – with this data, at least five *Pisolithus* species were distinguished (Anderson et al., 1998; Martin et al., 1988).

In 2001, Díez et al. reported at least 5 different species of *Pisolithus* in the Mediterranean region (Díez et al., 2001). In 2009, also 5 species were identified in Morocco (Yakhlef et al., 2009). Three years later, in 2012, the distinctiveness of the British, French and Swedish *Pisolithus* samples was confirmed through molecular and morphological studies, and a new species from Asia was found; in 2015, the presence of *Pisolithus tinctorius*, *Pisolithus arhizus* and *Pisolithus capsulifer* was reported in Macedonia (Phosri et al., 2012; Rusevska et al., 2015).

Rusevska et al. (2015) reported high similarity between *P. tinctorius*, *P. arhizus* and *P. capsulifer*, regarding the spore size and the peridium colours (Rusevska et al., 2015).

1.2. *Pisolithus tinctorius* – Bioactive Potential

Fungi have been known worldwide as producers of compounds with a wide range of capabilities. Some of the species can produce compounds with beneficial health properties such as antioxidants, anti-inflammatories, antivirals and even compounds that show ability to reduce cholesterol, control diabetes and autoimmune diseases such as rheumatoid arthritis and lupus, and that also reported antitumoral activity, more recently (Smith et al., 2002).

Like other basidiomycota fungi, *Pisolithus* does not fall short on expectations regarding multiple bioactive potential. In fact, *Pisolithus* have shown a considerable range of applications, whether they are in the field of environmental scene (agriculture and plants, for example) or in the context of health and potential future treatment of diverse present or emerging pathologies. Thus, the research on *Pisolithus* and their transversal biological and chemical potential deserves special attention.

Marx (1977) reported the presence of allelochemicals in this fungus, backed up by several studies regarding the interaction between *Pisolithus tinctorius* and its host plants – *P. tinctorius* showed substances that, when released, could affect the growth of other organisms (Marx, 1977). Notably, one of the areas of study regarding *Pisolithus* and their application contemplates this chemical exchange: its use on afforestation programs. This fungus is known to have the ability to create symbiotic relationships with host plants, and contribute to their faster and healthy growth, even if the environmental conditions are not the ideal for the plant in question – actually, the *Pisolithus* genus is proven to have the capability not only of creating a barrier and cancelling factors such as low soil fertility, high temperatures and unsuitable soil pH, but also of improving the uptake of nutrients like magnesium, phosphorus, sulphur, potassium and calcium. In addition, is also able to increase the concentration of monoterpenes in the plant, that can provide highest protection for the plant, avoiding infections at the radicular level caused by fungal pathogens (Abreu, 1987; Sugui, Alves de Lima, et al., 2003). It is used mainly in trees like *Pinus* and *Eucalyptus*, among other host plants that can represent a considerable economic value (Coutinho, 2004; Kope & Fortin, 1990; Oliveira et al., 2012).

Considering this background and given that fungi can be producers of compounds with biotechnological interest, other bioactive applications of *Pisolithus* were explored by some authors, mainly suggesting that this genus is a potential producer of compounds with pharmacological applications – *P. tinctorius* can be an alternative in the fight against several diseases and current pathologies, relying mainly on its potential as an antibiotic, fungicide and even as a possible cancer treatment (Baumert et al., 1997; Abreu et al., 1991; Gill, et al., 1989; Lobo et al., 1988; Zamuner et al., 2005).

Several authors reported the presence of compounds that can be found and extracted from *Pisolithus tinctorius* (Abreu et al., 1991; Gill et al., 1989; Lobo et al., 1988; Zamuner et al., 2005). Table 1 shows different compounds isolated by these authors and the origin of the samples.

Table 1: Compounds isolated from *Pisolithus tinctorius*

Compound	Biological Material	Related country	Reference
3-oxo-pisolactone	Fruiting bodies	Portugal	Lobo et al. (1988) De Abreu et al. (1991)
3-acetyl-24-methyl-lanostan-8,24-dien-22-acetate	Fruiting bodies	Portugal	Lobo et al. (1988) De Abreu (1991)
Pisosterol	Fruiting bodies	Portugal Australia Brazil	Lobo et al. (1985) Gill et al. (1989) Zamuner et al. (2005)
Methyl-pisosterol	Fruiting bodies	Portugal Australia	Lobo et al. (1985) Gill et al. (1989) Abreu (1991)
3-epi-pisosterol	Fruiting bodies	Brazil	Zamuner et al. (2005)
3B, 22, 23-trihidroxi-24-etil-lanostran-8, 24-etil-lanostan-8, 24(28)-dien-22-acetate	Fruiting Bodies	Brazil	Zamuner et al. (2005)
Pisolactone	Spores and Mycelium	Portugal Switzerland Mexico	Lobo et al. (1983) Baumert et al. (1997)
25-methyl-pisolactone	Mycelium	Switzerland México	Baumert et al. (1997)
22R-methyl-22R-lanostan-8,24(28)-dien-3B,22-diol	Mycelium	Switzerland Mexico	Baumert et al. (1997)
Lanosterol	Mycelium	Brazil	Zamuner et al. (2005)

Agnosterol	Mycelium	Brazil	Zamuner et al. (2005)
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1.3. Anticancer Activity

The main research of fungi as a source of anticancer compounds started in 1968, when Ikegawa et al. reported antitumor activity in Sarcoma 180 of an extract obtained from Polyporaceae, Phelinusceae and fungi from other families (Ikekawa et al., 1968). After this research, three important anticancer drugs were developed: krestin (from cultured mycelium of *Trametes* – *Coriolus versicolor*), lentinan (from fruiting bodies of *Lentinus edodus*) and scizophyllan (from *Schizophyllum commune*) (Ikekawa, 2001; Takashi Mizuno, 1999; Wasser & Weis, 1999).

Cancer is one of the leading causes of death globally, and according to the World Health Organization (WHO, 2015), it is responsible for the death of about 8.8 million people per year, and this number of diagnosed cases can have an increase of about 50% until 2030, reaching about 22 million cases. In the United States, cancer is the second leading cause of death among children from ages 1 to 14, with Leukemia being the most common cancer, followed by brain cancer and other parts of the nervous system (Jemal et al., 2009). The anti-cancer drugs currently available on the market demonstrate several aggressive side effects, in addition to the lack of selectivity, which may reflect the emerging need to find a new form of treatment that is both effective and less toxic to the patient's body (Leszczyniecka et al., 2001; Patel & Goyal, 2012). Today, conventional cancer-related therapies involve surgeries, chemotherapy, and radiation therapy, depending on the type of cancer and its development in the patient, resulting in irreversible damage and increased patient fragility (Chan et al., 2009; Silva, 2012).

For this reason, the search of natural methods that may involve less secondary effects has increased in the last years, leading to a special attention on the research regarding organisms like mushrooms, which already have been associated with anticarcinogenic activities in more than 50 species worldwide, with a particular detail: the high level of tolerance from the human body to their action and the non-harmful secondary effects on the organism (Kidd, 2000).

Different bioactive molecules, obtained from different mushroom species, have been identified and isolated, and some of them with antitumor substances (Mizuno, 1999; Wasser & Weis, 1999). At least 651 species of mushrooms revealed antitumor or immunostimulating polysaccharides, either naturally collected or artificially grown in pure culture mycelia or culture filtrate, being the Basidiomycetes proved to be of great importance in this field, since they were shown to contain biological possible active polysaccharides (Reshetnikov & Tan, 2001).

For several years, some studies have been carried out using *Pisolithus tinctorius*, in order to ascertain its chemical and biological potential and the presence of bioactive compounds that can be extracted from this species, and in fact some peculiarities have been found - the main one remotes to the fact that *Pisolithus tinctorius* show anticancer potential. Initially, in this field, Gill et al. (1989) opened a range of future research on the biological potential of this organism, having isolated the compound which was named pisosterol (Figure 1), and was later targeted on several studies by Montenegro et al., one of the main pioneers of the research on *Pisolithus tinctorius* and its associated compounds (Montenegro et al., 2008; Gill et al., 1989; Montenegro et al., 2004)

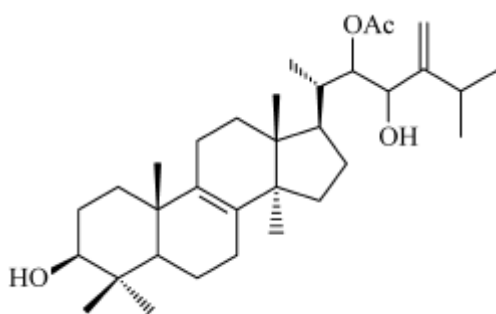


Figure 1. Chemical structure of pisosterol, isolated by Gill et al. (1989)

In 2004, Montenegro et al., based on studies by Coker & Couch (1928), published their first study regarding the pisosterol, the triterpene isolated from *Pisolithus tinctorius*. The samples were collected from the Northeast region of Brazil and the isolated compound was tested on three different animal cell models: mouse erythrocytes, sea urchin embryos and tumor cells, with the objective of evaluating the cytotoxicity of pisosterol, measuring the inhibition of sea urchin eggs

development, the lysis of mouse erythrocytes and the proliferation of tumor cell lines. In one hand, pisosterol had no activity on mouse erythrocytes, showing no membrane damage, and no activity on sea urchin eggs, showing no inhibition on their development. On the other hand, it showed a strong growth inhibition of the seven tumour cell lines tested. In addition, the activity of pisosterol was compared to two chemotherapy drugs: doxorubicin and etoposide. The activity of these two compounds was compared to pisosterol activity, regarding the IC50 values, and pisosterol showed only a slightly lower potency (Montenegro et al., 2004).

Three years later, in 2007, Montenegro et al. continued their investigation regarding pisosterol, and published a study in order to determine whether the antiproliferative effects observed for pisosterol were related to cell differentiation induction, using human promyelocytic leukemia cells (HL-60), investigating also the role of this compound in proliferation and apoptosis induction after differentiation. Also, the effects of pisosterol on normal human cell lines were evaluated, using peripheral blood mononuclear cells (PBMC). The results showed a reduction in cell viability in a time and dose-dependent manner, since it was reported a decrease in cell viability in the presence of pisosterol with increasing exposure time. Also, microscopic examination of the cells treated with pisosterol demonstrated morphological changes, indicating that this compound induces HL-60 differentiation. Regarding the normal human cell line tested (PBMC), no cytotoxicity was detected in the presence of pisosterol: this can prove that the triterpene may be selective for tumour cells and thus, a promising heal substance (Montenegro et al., 2007).

In 2008 the same authors, Montenegro et al., evaluated the *in vivo* antitumor activity of pisosterol, regarding histopathological and morphological analyses of the Sarcoma 180 tumor cell lines transplanted in mice, and the organs like liver, spleen and kidney were also analysed, in order to evaluate toxicological aspects of the pisosterol action on the organism. After 7 days of expose, the antitumor activity of pisosterol was confirmed, with tumor growth inhibition ratios of 43.0% and 38.7% regarding Sarcoma S180 transplanted-mice treated with pisosterol at 50 and 100 mg/ml, respectively. The liver, kidney and spleen histopathological evaluation demonstrated that liver and kidney were affected by pisosterol, but these lesions and alterations could be reversible (Montenegro et al., 2008).

Following the studies made by Montenegro et al., Burbano et al. (2009) conducted a study in a HL-60 cell line, analysing GTG-banded chromosomes before and after treatment with pisosterol, in order to understand the mechanisms of action of this compound (Burbano et al., 2009). HL-60 cell genome contains an amplified proto-oncogene (C-MYC) that is a regulator of the cell cycle, controlling the cell growth, differentiation, apoptosis and neoplastic transformation, and there is a relation between aberrations on homogeneously staining regions (HSR) and the amplification of C-MYC, located at 8q24, in HL-60 cell line. Pisosterol was induced at three different concentrations (0.5, 1.0 and 1.8 $\mu\text{g/ml}$) and, before the treatment, 99% of the cells showed the HSR 8q24 aberration; although, after the treatment with 1.8 $\mu\text{g/ml}$ of pisosterol, only 10% of the cells maintained this aberration. In addition, the cells treated with pisosterol were washed and re-incubated in the absence of the compound, and only 30% of the analysed cells lacked the HSR 8q24 aberration, demonstrating the possibility of a Pisosterol ability to block the cells at interphase. Additionally, pisosterol also inhibited cell growth at a concentration of 1.8 $\mu\text{g/ml}$. Making a bridge between this study and the one carried out by Montenegro et al. (2004), these results were also compared with those found for doxorubicin: cells that do not demonstrate a high degree of HSRs gene amplification are easy targets for chemotherapy, having a less aggressive and invasive behaviour. Pisosterol has a slightly less potency comparing to doxorubicin and can block HSR amplification, making this a promising compound to test in combination with some of the usual anti-cancer substances (Burbano et al., 2009; Montenegro et al., 2004).

More recently, in 2011, Pereira et al. conducted a study on glioblastoma multiforme (GBM) cell lines U343 and AHOL1, regarding the morphological and cytogenetic characteristics of this cell lines before and after the treatment with pisosterol (Pereira et al., 2011). This compound did not induce any morphological alteration, suggesting that pisosterol does not cause cell differentiation in GBM cell lines – contrary to what happens in HL-60 cells on the studies carried out by Montenegro et al., (2007). In addition, at a concentration of 1.8 $\mu\text{g/ml}$ of pisosterol, the compound was able to block the cells with C-MYC gene amplification. Associating this study with the one made by Burbano et al., (2009) pisosterol can be used on the reduction or total inhibition of C-MYC amplification, making the cancer cell lines an easier target on the fight against this disease.

In 2015, Alves et al. verified also the presence of an anticancer potential regarding *Pisolithus tinctorius* species, conducting a study using *P. tinctorius* spores and testing their cytotoxic activity on three cancer cell lines (human osteocarcinoma cell line – MG63, human breast carcinoma cell line – T47D and human colon adenocarcinoma cell line – RKO) and one normal human cell line (human brain capillary endothelial cell line (hCMEC/D3), through a fractionation (Alves et al., 2015). After 48h of exposure to the extracts, only 5% of the cancer cells were viable in 4 of 11 fractions, and a low impact on the normal cells was registered. This data shows that, apart from other structures of *P. tinctorius* species, the spores also might be a powerful and interesting source of anticancer compounds.

In a global view, the studies carried out by Montenegro et al. (2004, 2007, 2008), Burbano et al. (2009), Pereira et al. (2011) and Alves et al. (2015) as described above, show that *Pisolithus* may produce compounds that can be isolated in order to create new anti-cancer therapies.

1.4. Antimicrobial Activity

The first antimicrobial substance produced by fungi was discovered in 1928 by the British scientist Alexander Fleming, when he noticed an inhibition of *Staphylococcus aureus* growth through the mold: penicillin was isolated for the first time from the fungus *Penicillium notatum*, now named as *Penicillium chrysogenum* (Takahashi & Lucas, 2008). The isolation of antimicrobial agents started to be the most common procedure to tackle bacterial infections, and the use of these compounds started to create a new problem: the increase of bacterial resistance, since 1990. The newest findings on this field, during 1990s, started to show less efficiency on the search for new antimicrobial compounds, creating the necessity of development by the pharmaceutical industry and also increasing the needs of possible sources of products capable to fight new resistant bacteria (Guimarães et al., 2010; Livermore et al., 2011). Actually, increasing resistance to antimicrobials is a major public health problem and thus, there is a necessity to keep a continuous search for new bioactive compounds, being imperative to find effective compounds against diverse pathogens (Tenover, 2006).

Studies conducted by Marx (1970) were the initial step on the finding of antagonistic properties of mycorrhizal fungi against pathogenic fungi and soil bacteria, reporting the production of antibiotics by over 100 basidiomycete mycorrhizal fungi (Marx, 1977).

Nowadays, secondary metabolites are associated with the biotechnological exploration, regarding the search for new antibiotics by the pharmaceutical industry. Most of these compounds are obtained through fungi, such as penicillin, steroids or even cyclosporine (Coelho & Ribeiro da Silva, 2006). The basidiomycetes are considered a good source of these compounds, being associated with antimicrobial potential since 1941, when pleuromutiline - a diterpene available on the market and used in the treatment of animal infections - was isolated and characterised for the first time. With a wide range of fungi that were tested since this decade, basidiomycetes are still one of the major sources of antimicrobial compounds and are receiving more and more attention on this subject, supported by the development and increase of scientific technologies (Maziero et al., 1999; Suay et al., 2000; Anke, 1989).

Regarding the antifungal activity of *P. tinctorius*, Kope and Fortin (1989) showed that this species was able to produce metabolites in agar medium that caused hyphal lysis and other morphological changes of phytopathogens, followed by a cellular dysfunction (Kope & Fortin, 1989). In 1990, the same authors characterised the cell-free culture filtrate on the hypha and conidia of different phytopathogenic fungi, like *Rhizoctonia praticola*, *Truncatella hartigii* and *Sphaerospora brunnea* - used in the hyphal morphology assay - and *Cochliobolus sativus*, *Fusarium solani* and *T. hartigii*, for conidial production, maintaining also two strains of *Brunchorstia pinea*. This research demonstrated that *P. tinctorius* was able to release a metabolite that lysed hypha and conidia and also inhibited germination of conidia, showing a complete growth inhibition for *R. praticola*, *T. hartigii* and *S. brunnea*, a conidial germination inhibition for *F. solani*, *T. hartigii*, *B. pinea*, and *C. sativus* and conidial lysis for *T. hartigii*, *B. pinea* and *C. sativus*. These data was able to demonstrate the potential of *Pisolithus tinctorius* metabolites against a wide range of fungi (Kope & Fortin, 1990).

Tsantrizos et al. (1990) isolated and characterised two compounds found in *Pisolithus tinctorius*, that demonstrated antibiotic potential: p-hydroxybenzoylformic acid [2-(4'-hydroxyphenyl)-2-oxoethanoic acid, 1] and (R)-(-)-p-hydroxyethanoic acid, 2], being named as pisolithin A and pisolithin B, respectively (Tsantrizos et al, 1991). These compounds showed a strong antifungal activity when used against diverse phytopathogenic fungi, such as *Phytophthora* sp., *Rhizoctonia solani*, *F. solani*, *Puthium debaryanum*, *Pythium ultimum*, *Verticillium dahliae*, *Pyrenochaeta terrestris*, *C. sativus*, *Septoria musiva* and *B. pinea*, that are the cause of large economic losses on tree nurseries, and dermatogenic fungi like *Microsporum gypseum* and *Trichophyton equinum*, that cause dermatitis on animals and humans. The two compounds caused also inhibition of mycelial growth and hyphal lysis on *T. hartigii* in concentrations ranging from 50 to 150 µg/ml, inhibiting also chitin synthesis within 12 to 24 h of exposure to both Pisolithin A and B. The chemical structure of pisolithin A and B is presented in Figures 2 and 3, respectively.

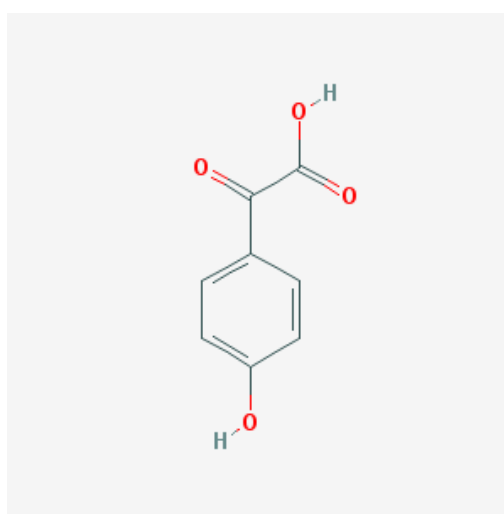


Figure 2. Chemical structure of pisolithin A

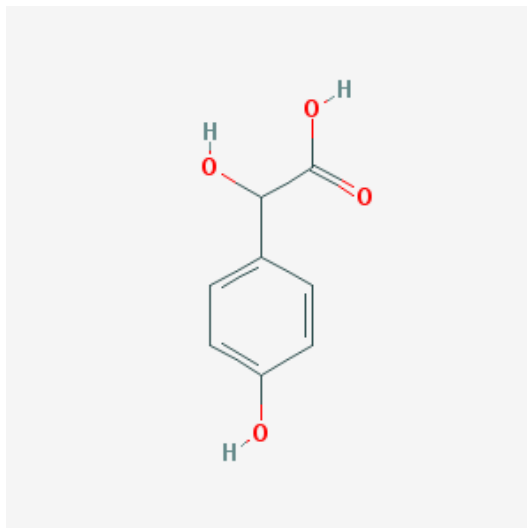


Figure 3. Chemical structure of pisolithin B

It is necessary to underline the fact that the inhibition caused by these compounds was compared to other antifungal chemical agents such as nystatin and polyoxin D, and the result was a higher inhibition when pisolithin was used (Tsantrizos & Ogilvie, 1991).

Shrestha et al. (2005) reported the ability of extracts of *Pisolithus* sp. to inhibit the growth of *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in a concentration of 10 mg/ml, and also a low inhibition of *Staphylococcus aureus* growth (Vaidya, Shrestha, & Wallander, 2005). In 2011, Ameri et al. tested the anti-staphylococcal activity of crude extracts of two strains of *Pisolithus albus* collected from Pune, India. Distinct organic solvents were used for extraction of secondary metabolites, of increasing polarity, such as ethyl acetate, chloroform and methanol, and the extracts were tested against 30 strains of methicilin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *Staphylococcus aureus* (MSSA). Although all strains showed resistant to the chloroform extract, strong activity was reported for ethyl acetate and methanol extracts. In relation to the bioactive components, diterpenoids, triterpenoids, sesquiterpenoids and polysaccharides were bioassayed, regarding the antimicrobial activity: while triterpenoids and diterpenoids showed weak to moderate activity and polysaccharides demonstrated weak efficacy, sesquiterpenoids extract resulted in a maximum effectiveness (Ameri et al., 2011).

In 2015, Mohan et al. tested the inhibition caused by eight different ectomycorrhizal fungi, including *Pisolithus albus*, in nine distinct plant pathogenic fungi (Mohan et al., 2015). The eight ECM fungi demonstrated antagonistic efficacy against plant pathogenic fungi, and *Pisolithus albus* showed a percentage of inhibition from 12.22% to 47.78%, depending on the pathogenic tested, revealing also chitinase production. This study was able to show that, given the antagonistic potential of ECM fungi, like *Pisolithus albus*, this species can be used as bio-fungicide in tree nurseries and plantations. Additionally, this fungus can be also used by industries for chitinase production, in order to develop biofertilisers and other products.

All these data can lead to a clue that *Pisolithus* can produce compounds with considerable activity and antimicrobial potential, reinforcing the importance of continuing the research on this field, on the pursue of new possible antibiotics and antifungal products.

1.5. Aim of the dissertation

The main purpose of this work was to evaluate the bioactive potential of the *P. tinctorius* mycelium and fruiting body regarding cancer cell lines for anticancer activity and bacteria strains for antimicrobial activity. Considering this main goal, the following specific tasks were defined:

- to isolate *P. tinctorius* and to grow the fungal mycelium in culture medium;
- to prepare mycelium 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.

2. Materials and Methods

2.1. *Pisolithus tinctorius* harvesting

Specimens of *P. tinctorius* (Figure 4) were collected in a forest ecosystem in northern Portugal. The samples were collected and kept in a box, covered with absorbent paper and then cleaned on the surface using dry paper, to eliminate as much dust and dirt as possible, but always keeping its surface intact.



Figure 4. *Pisolithus tinctorius* samples

2.2. Choice of culture media

Due to the lack of variability of media composition, many microorganisms are still unculturable, and the choice of a culture media is one of the most important prerequisites, due to the huge variety of microorganism's growth requirements, like nutrients, pH and other parameters (Bhattacharya, Vijayalakshmi, & Parija, 2002). The preparation of a suitable culture media was the first step for the isolation and growth process of the *P. tinctorius* mycelium.

Usually, media containing high carbohydrate and nitrogen sources are required for the growth of fungi, mainly at a pH range from 5 to 6, and a temperature between 15 to 37°C. Since there are two types of agar, natural and synthetic, the synthetic type was chosen because, with this one, the process of repeating the same culture conditions is facilitated, because the synthetic medium has the entire composition previously defined.

Firstly, three different media were tested: Malt Extract Agar (MEA), Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA). In order to evaluate the best culture media to use on the growth of *P. tinctorius* mycelium, the diameter of the mycelium was evaluated for each medium after an incubation of the petri dishes in 20 days at 25°C and, in addition, the contamination rate (number of contaminated plates in relation to the total number of plates used) was also evaluated on the isolations using the three different media.

In an attempt to decrease the rate of contamination of the petri dishes, 10 ml of Penicillin-Streptomycin (10,000 U/ml) was added to PDA medium (10 ml/L), testing the isolation in petri dishes containing media with and without this antibiotic. The contamination rate was reported by counting the number of plates that had contamination in relation with the number of plates that had no contaminations.

2.3. Inoculation and incubation of the *P. tinctorius* mycelium

After cleaning the surface of the specimens with dry paper to avoid any dirt or dust, the samples were manually opened without any invasive method or cutting material to separate each sample in two halves, in order to avoid the contamination of the interior of the fungal body. Once opened, a sterile scalpel was used to cut small fragments from the inside, about 2 to 5 mm each, which were then placed in the petri dishes containing the growth medium. One to three fragments were placed on each plate, approximately in the centre of the plate, spaced around 2 to 4 cm apart. Then, following the incubation technique of De Araujo et al., (2000) and Coker & Couch (1928), the plates were wrapped in Parafilm and incubated at 25°C in dark. In the present study, the temperature factor was also tested by placing half of the plates at room temperature and others at 25°C.

The fungus was subcultured every 15 to 20 days using the same procedure described above. A sterile scalpel was used to cut a small fragment of the mycelium, and then transferred to a new plate.

Figure 5 presents a scheme of the isolation and replication processes.

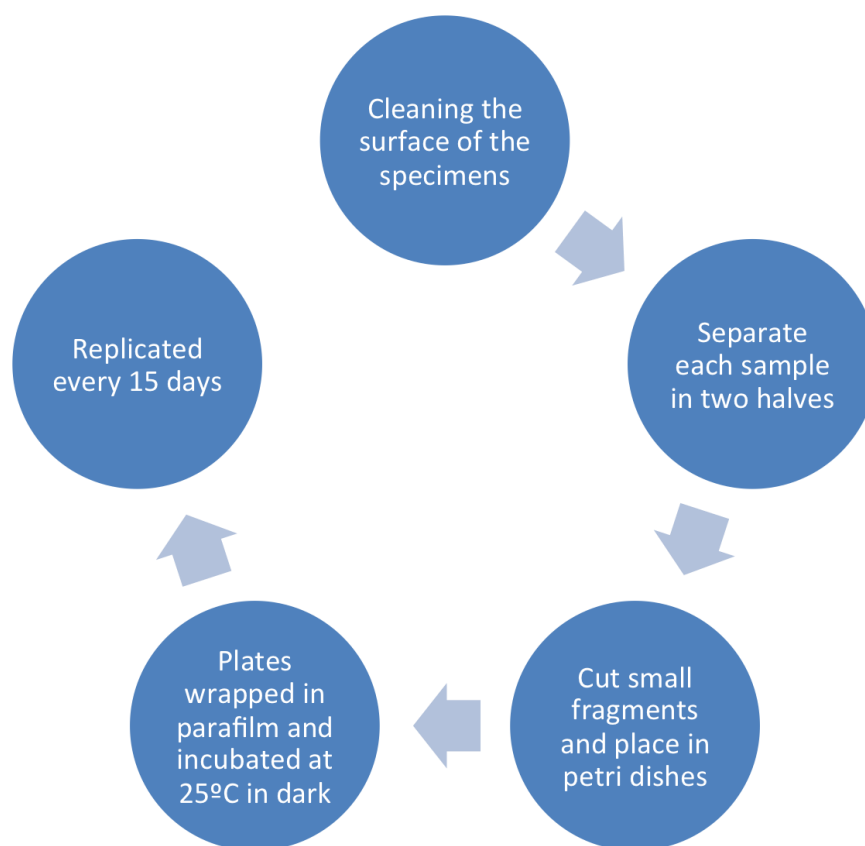


Figure 5. Scheme of the isolation and subculturing processes

From one to three days after placement of a portion of *Pisolithus* on a petri dish, it was possible to verify the start of the colour change of the culture medium used, and this fact was also reported, categorising the colour from 1 to 4, according to the intensity of the colour, from the normal colour of the media (1), to yellow (2), brownish (3) and dark (4).

The mycelium diameter of the isolates was also measured after 5, 10, 15 and 20 days, regarding the period of incubation, in order to evaluate the *P. tinctorius* mycelium growth in the present experimental conditions.

After the mycelium of the *P. tinctorius* has been properly grown for 20 days, both the mycelium and surrounding dyed agar were transferred into flasks, freezed at -80 °C and lyophilised. Freeze dried material was stored at -80 °C for subsequent extraction.

2.4. *P. tinctorius* isolation - the fruiting bodies

The same process of cleaning the surface of the fungus was done, as explained before. Then, the samples were opened using the same procedure, and the interior was cut and transferred using a sterile scalpel to a flask. To avoid the loss of the viability of the compounds, the samples were frozen at -80 °C. The biomass was then lyophilised and stored also at -80 °C.

2.5. Extraction

2.5.1. Selection of the best extraction solvents

Considering that data about the extraction of *P. tinctorius* mycelium is scarce, different solvents were used in order to decide which one was the most promising for the extraction of compounds namely in terms of the extraction yield and biological activity. In this sense, four solvent mixtures or solvents were tested: Dichloromethane/Methanol (DCM/MeOH-2:1), Ethyl-acetate/Methanol (EtOAc/MeOH-2:1), Methanol (MeOH) and Dichloromethane (DCM).

From the lyophilisation process 2 g of biomass obtained from the mycelium and the fruiting bodies were extracted with 75ml of each solvent mixture or solvent. On a first step, 25ml of each solvent mixture/solvent was added, and sonicated for 30 s to force cell lysis; then, the solutions were centrifuged at 7000 rpm for 10 min. Supernatants were transferred to a clean flask and the pellet reextracted. This process was repeated three times, using a total of 75ml of solvent.

The extracts were evaporated on a rotatory evaporator with reduced pressure (BUCHI Rotavapor R-210) and transferred to glass vials, previously weighed, in order to calculate the yield of the extraction.

The crude extracts obtained from the extractions were assayed for cytotoxic activity in cancer and normal cell lines, in order to select the most promising for the isolation of bioactive compounds.

2.5.2. The final extraction

According to the cytotoxic results obtained with the crude extracts, the solvent mixture DCM:MeOH was selected for further studies. Extraction was performed according to the extraction's flowchart presented in Figure 6.

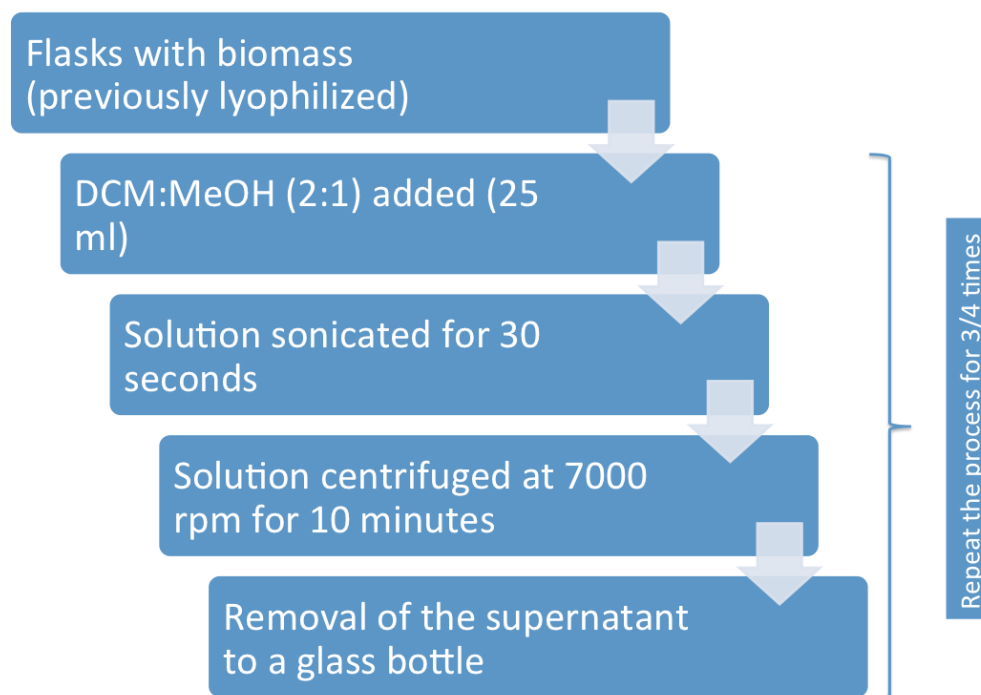


Figure 6. Extraction's process flowchart

Briefly, 25.3g of dry biomass of the mycelium were extracted with a total volume of 75ml of Dichloromethane-Methanol (2:1). After the three sequential extractions and if the supernatant had some colouration, a fourth extraction was done; Supernatants from the sequential extractions were combined and dried in a rotatory evaporator system. In order to prevent degradation during extraction, light exposure was minimised and temperature kept below 40° C. The extracts were stored at -20° C.

2.5.3. Extract fractionation

Crude extract fractionation was performed using Vacuum Liquid Chromatography (VLC). The all process was based on Edwards et al. (2004).

The vacuum used on this process allowed a very tight packing of the silica (the sorbent used). The silica used was the Silica Gel 60, which corresponds to 0.015-0.040 mm mesh silica.

The crude extract was fractionated by using a gradient of solvents, from hexane to EtOAc and MeOH, consecutively, that is from non-polar solvents to highly polar ones, being the last step the use of 100% MeOH. The VLC apparatus was composed by a Buchner funnel, a glass frit, a 24/40 adapter, a collection vessel, a three-way stopcock and a vacuum pump connected to the system as presented in Figure 7.



Figure 7. Vacuum Liquid Chromatography system

On a first step the silica gel was added to the filtration funnel, almost until the column was filled, leaving around 1 to 2 cm to the edge of the column. After this, the vacuum was turned on to compact the silica, and a mixture of Hex:EtOAc (9:1) was added to soak the silica. This process was repeated until the column was packed. A Whatman filter paper was placed on top of the silica, leaving always a minimum amount of solvent to keep it solvated.

The crude extract was suspended in Hex:EtOAc (9:1) and transferred to the top of the column, using a Pasteur pipette. The vacuum was turned on again to load the sample into the column. The fractions were collected into different flasks, using the solvent mixtures and the volumes presented in Table 2. The flasks were changed in order to fractionate the extract according to their polarity, from A (lowest polarity) to I (highest polarity) in a total of 9 fractions.

Table 2 – Solvents for the VLC fractionation procedure.

Fraction	Solvent Mixture	Volume (ml)
A	10% EtOAc 90% Hexane	300
B	30% EtOAc 70% Hexane	250
C	40% EtOAc 60% Hexane	250
D	50% EtOAc 50% Hexane	250
E	60% EtOAc 40% Hexane	250
F	100% EtOAc	250
G	90% EtOAc; 10% MeOH	250
H	75% EtOAc 25% MeOH	250
I	100% MeOH	600

The fractions were transferred to vials (previously weighted), and solvents were evaporated on a rotatory evaporator with reduced pressure. The dried fractions were weighted and stored at -80°C. All fractions were tested for anticancer potential using cancer and normal cell lines. The fraction with more interesting result was submitted to a High Pressure Liquid Chromatography (HPLC) to evaluate its chromatographic profile, evaluating its complexity and establishing the number of main components.

The HPLC was done using a Waters 1525 Binary HPLC Pump, with a normal phase Luna 5u Silica 100A 250 x 4.60 mm column, with an isocratic mixture of 75%

EtOAc and 25% MeOH and a 1.0 ml/min flow, with a dual slit UV/Vis absorbance detector set at 280 nm and 254 nm.

2.6. Nuclear Magnetic Resonance (NMR)

¹H Nuclear Magnetic Resonance analysis was carried out for the fractions that showed highest toxicity in cancer cell lines and highest viability on the normal human cell line, in order to obtain information about their complexity and some characterization of the compounds present in each fraction that are potentially responsible for the bioactivity. The samples were sent to Centro de Materiais da Universidade do Porto (CEMUP) and analysed using a “Bruker Avance III HD 400 MHz, 9.4 Tesla”.

2.7. Cytotoxicity assay

2.7.1. Cell cultures

A cytotoxicity assays using the crude extracts and fractions obtained from both the mycelium and the fruiting body of *P. tinctorius* were performed against the cancer cell lines colon adenocarcinoma cell line (RKO), human breast cancer cell line (T47D) and the human liver hepatocellular carcinoma cell line (HEPG2). The cytotoxicity was also evaluated using the normal human dermal micro vascular endothelial cell line (hCMEC/D3). The RKO and HEPG2 cell lines were obtained from the American Type Culture Collection (ATCC), T47D cell line was obtained from Sigma-Aldrich and the hCMEC/D3 cell line was kindly donated by Dr. PO Couraud (INSERM, France).

All the cell lines were grown in DMEM Glutamax medium, supplemented with 10% fetal bovine serum (FBS), 2.5 µg/ml fungizone and penicillin-streptomycin (100 IU/mL) and incubated at 37°C with a 5% CO₂ humidified atmosphere, being the culture medium changed with two days frequency.

2.7.2. MTT Assay and Statistical Analysis

For cell cytotoxicity assays, extracts or fractions were dissolved in DMSO, at a concentration of 10 mg/ml.

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to test the cytotoxicity of the extracts. Cells were seeded at a cell density of 3.3×10^4 cel/ml, on 96 wells plates. After 24h of cell adhesion, new medium was provided with the crude extract and the different fractions at a concentration of 100 µg/ml, 10 µg/ml and 1 µg/ml. Cells were incubated for 24, 48 and 72 h at 37°C and 5% CO₂. After each incubation time 20 µl of a MTT solution was add at a final concentration of 0.02 µg/ml and cells incubated for more 3 h. After incubation, the cell medium was discarded and 100 µl of DMSO were added, in order to solubilise the formazan crystals. As negative control, cells were exposed to medium with 1% DMSO and as positive control to 20% DMSO. All assays were run in triplicate. The optical density (OD) was measured using a microplate reader (Bio-tek Synergy™ HT), at 550 nm, and the parameter “Cell Viability” was used to express the cytotoxicity (considering 100% viability in the control). The program used to read the microplates was the Microsoft BioTek Gen5 Data Analysis Software.

The full methodology scheme is presented in Figure 8.

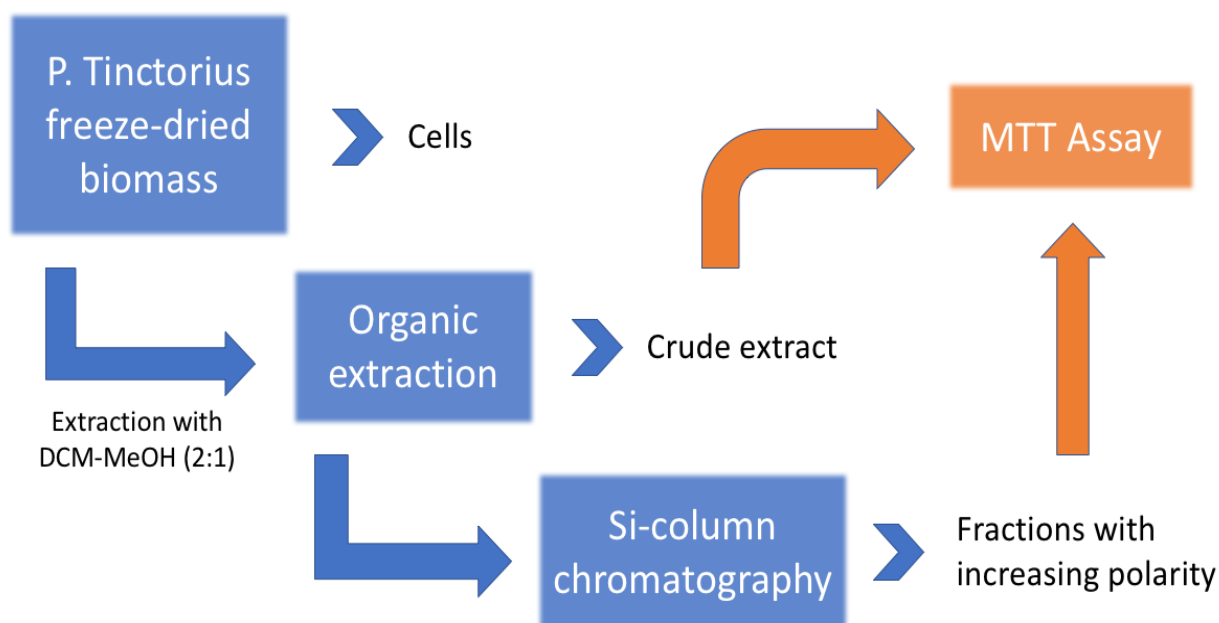


Figure 8. Full methodology scheme

Microsoft Office Excel was used to calculate averages and standard deviations and to present the results graphically.

2.8. Antimicrobial activity: Agar disk-diffusion method

The crude extracts of *P. tinctorius* mycelium and fruiting body, obtained with the 4 solvents tested before - Dichloromethane/Methanol (DCM/MeOH-2:1), Ethyl-acetate/Methanol (EtOAc/MeOH-2:1), Methanol (MeOH) and Dichloromethane (DCM) and the 9 fractions (A to I) were evaluated through the agar disk-diffusion method.

The antimicrobial screening susceptibility assay was done using the disc diffusion method. *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922) and *Salmonella typhimurium* (ATCC 25241) bacterial strains and *Candida albicans* (ATCC 10231) were grown in plates in Mueller-Hinton agar (MH) at 37°C.

To prepare the bacterial and *Candida albicans* MH plates, pure colonies were picked from cultures in MH using a swab, and suspended in 5 ml of buffered peptone water. The turbidity of the inoculum was then adjusted in order to equal a 0.5 McFarland standard (absorbance from 0.8 to 1, at a wavelength of 600 nm).

Blank discs of 6 mm diameter were placed in the inoculated plates and impregnated with 15 µl of a 1 mg/ml solution (in DMSO). Blank discs with 15 µl DMSO were tested as a negative control. An example of the test can be found in Figure 9.

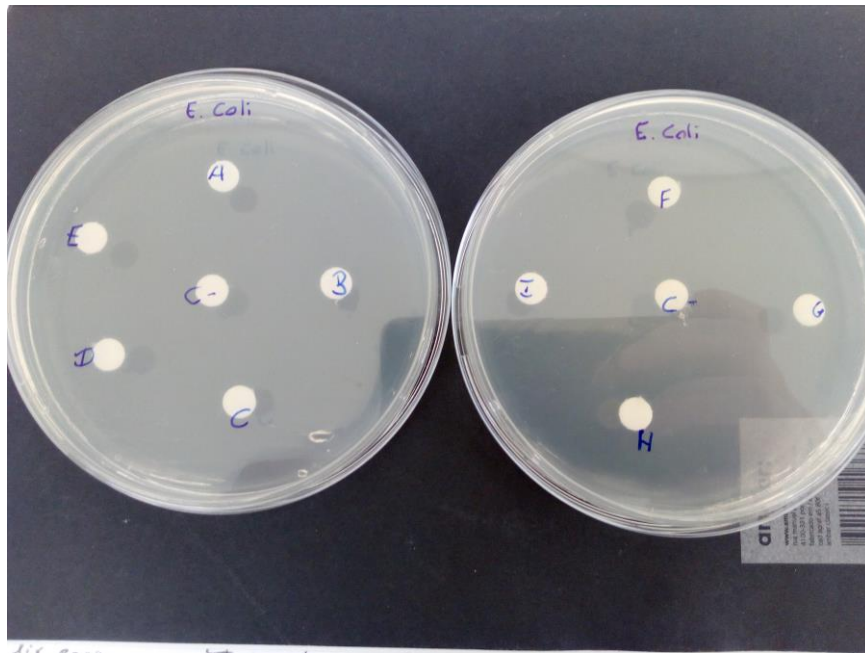


Figure 9. Example of the Agar disk diffusion method

Plates were left 30 min at room temperature and then incubated during 24h at 37°C.

The antibacterial activity is noticed when a halo with a determinable diameter is visible around the disc.

3. Results

3.1. Isolation and growth of the *P. tinctorius* mycelium

The mycelium diameter was measured, in order to analyse which media was more efficient on the *P. tinctorius* growth.

Cultures containing MEA media showed the lowest mycelium growth, with an average mycelium diameter of 17.1 mm, followed by cultures with SDA media, with an average diameter of 25.3 mm. PDA demonstrated to be the best media among the three, revealing an average mycelium diameter of 45.6 mm.

Thus, after several attempts to isolate the fungus in different culture media (MEA, SDA and PDA), the Potato Dextrose Agar media showed the best growth rate (relation between the growth of *Pisolithus* in the petri dish and the time of

growth) and, in addition, was the one that had the lowest contamination rate, counting the number of contaminated plates in relation to the total number of plates used.

After choosing Potato Dextrose Agar as the best media to use for the *P. tinctorius* isolation and growth, the average mycelium diameter was measured, using 10 samples for each period of incubation, corresponding to 5, 10, 15 and 20 days. Figure 10 presents the mycelium growth in relation to the period of incubation.

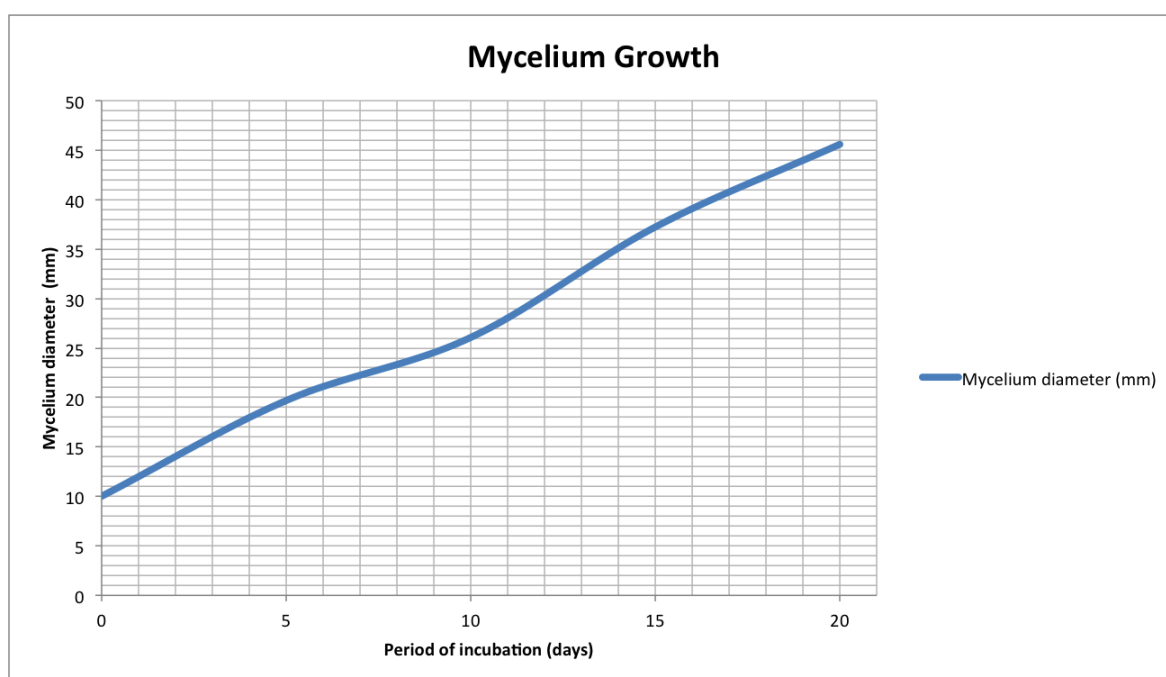


Figure 10. *Pisolithus tinctorius* mycelium growth in 20 days period incubation

It is possible to notice a crescent growth of the mycelium in each period of incubation. At 5-day incubation, the average diameter corresponds to 19.7 mm, followed by 26.1 mm at 10-day incubation, 37.2 mm at 15-day incubation and finally, an average of 45.6 mm at the maximum period of incubation time (20 days).

It is important to highlight that the cultures did not revealed any growth at room temperature, starting only to demonstrate significant growth when incubated at 25 °C.

On a first isolation attempt, using the three culture media tested (MEA, SDA and PDA), the contamination rate was also evaluated, regarding the number of contaminated plates in relation to the total number of plates used. Figures 11, 12 and 13 show the contamination rate of the isolation using MEA, SDA and PDA, respectively.

- Using MEA as the culture media, 14 cultures were done, being 5 contaminated and 9 without any contamination, corresponding to 36% and 64%, respectively (Figure 11);

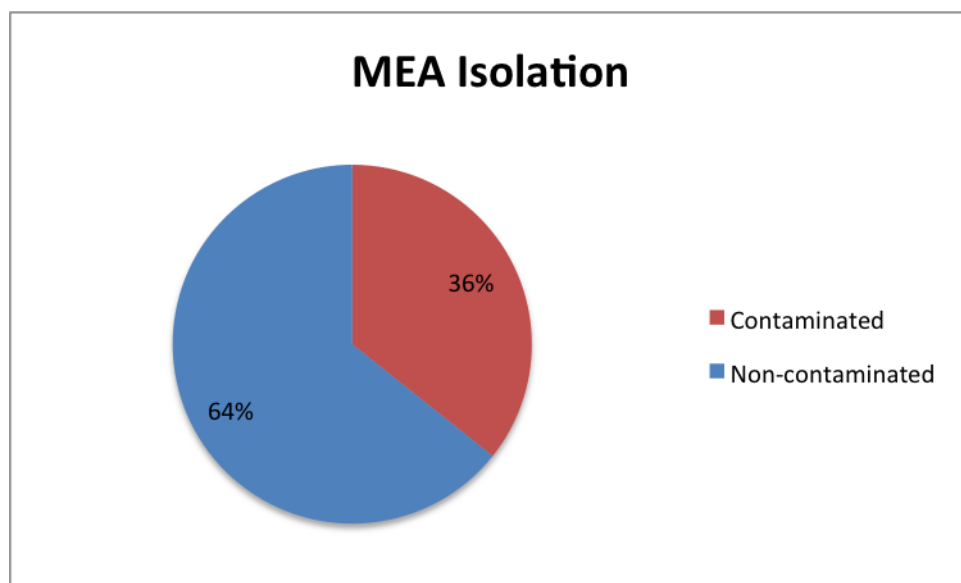


Figure 11. Contamination rate of *Pisolithus tinctorius* isolation using MEA media.

- Using SDA as the culture media, 12 cultures were done, being 9 contaminated and 3 without any contamination, corresponding to 75% and 25%, respectively (Figure 12);

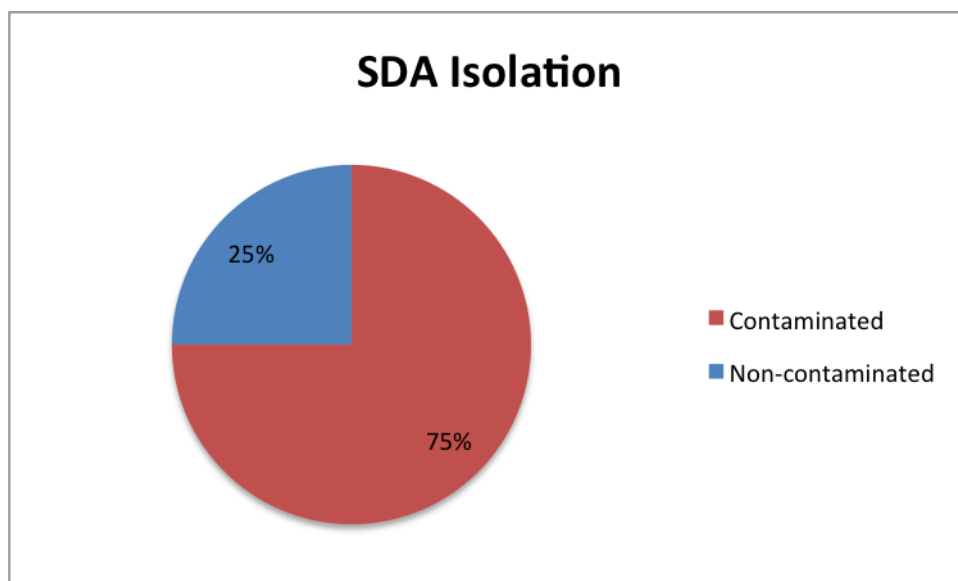


Figure 12. Contamination rate of *Pisolithus tinctorius* isolation using SDA media.

- Using PDA as the culture media, 24 cultures were done, being 4 contaminated and 20 without any contamination, corresponding to 17% and 83%, respectively (Figure 13);

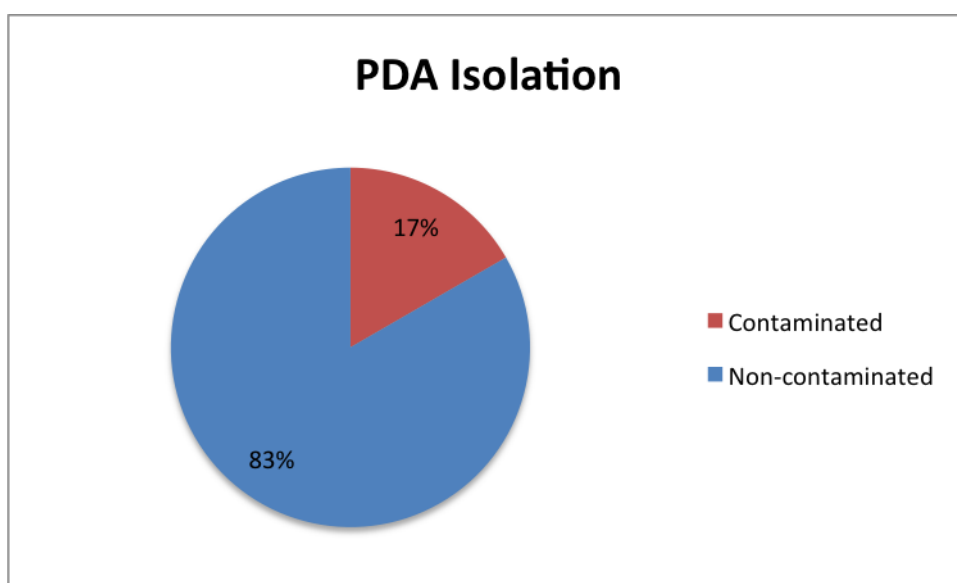


Figure 13. Contamination rate of *Pisolithus tinctorius* isolation using PDA media.

These results show that the isolation with PDA showed the lowest contamination rate, with only 17% of the plates contaminated, against 36% of contamination rate

using MEA. SDA showed the highest contamination rate, since 75% of the isolates had shown contamination.

3.2. Antibiotic influence – contamination rate

On an initial isolation, the influence of the penicillin-streptomycin antibiotic was tested, placing 10 ml of Pen-strep in 1L of medium and using medium without any antibiotic for an initial isolation, in order to obtain a contamination rate – a percentage of the contaminated cultures in relation with the non-contaminated ones. 71 samples were initially isolated, being 47 with the presence of Pen-strep and 24 without the antibiotic. After 2 to 5 days, the following was observed:

- 4 petri dishes were contaminated and 20 petri dishes were not, without the presence of antibiotic;
- 25 petri dishes were contaminated and 22 were not, with the presence of antibiotic.

Figures 14 and 15 represent the contamination rate of the isolations, in percentage.

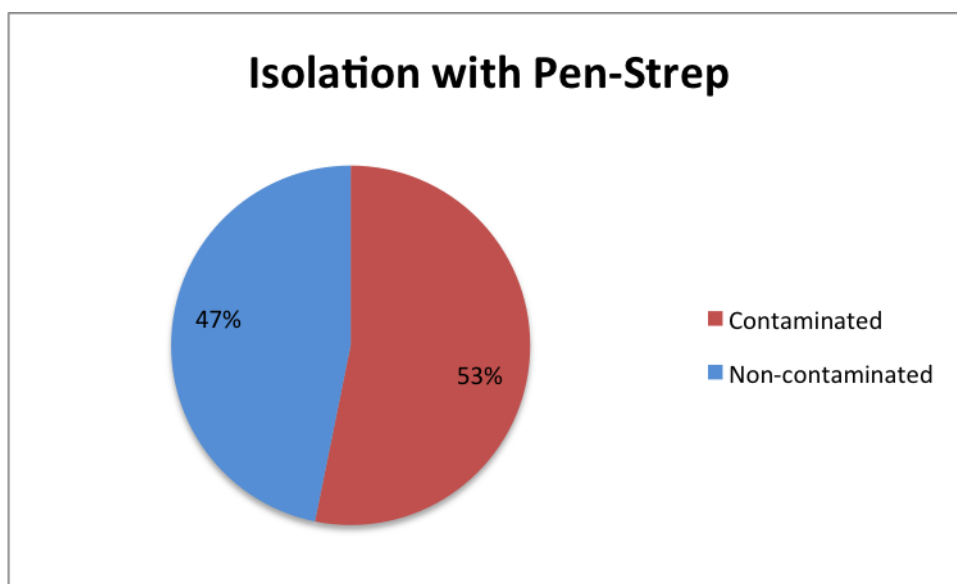


Figure 14. Contamination rate of *Pisolithus tinctorius* isolation using penicillin-streptomycin.

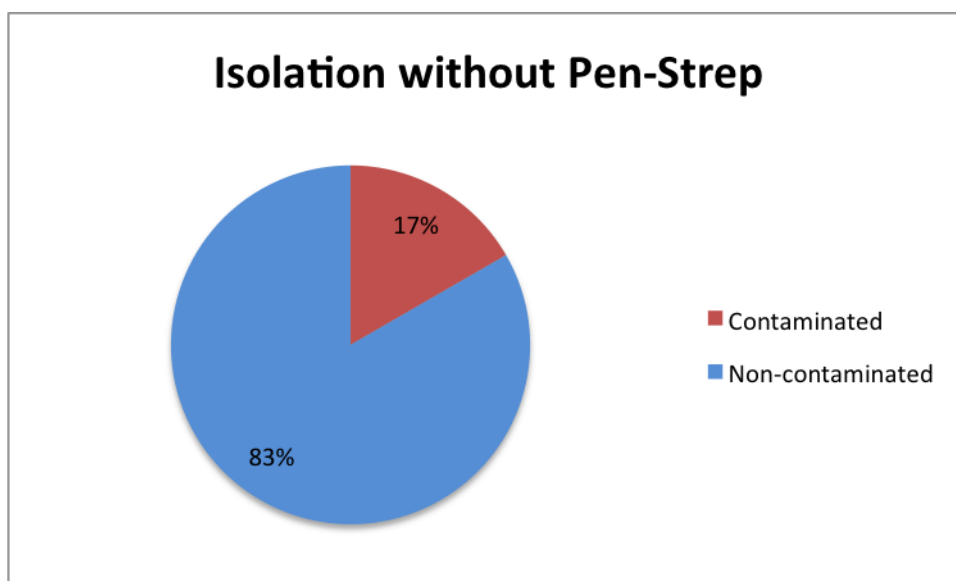


Figure 15. Contamination rate of *Pisolithus tinctorius* isolation without the use of penicillin-streptomycin.

After analysing some of the petri dishes visually, it was possible to see, mainly yeast and filamentous fungi contaminations, as shown in Figures 16 and 17.



Figure 16. Yeast contamination of *P. tinctorius* culture



Figure 17. Filamentous fungi contamination of *P. tinctorius* culture

3.3. Subcultures – contamination rate

Pisolithus was subcultured every 15 to 20 days into new PDA agar plates. 4 main subcultures were followed visually after 2 to 5 days after the transference of the mycelia to a new petri dish, and the number of contaminated and non-contaminated subcultures was reported, in order to obtain a relation between these two data. Each subculture was done on the same day and at the same conditions. The following data was observed:

- On a first main subculture, 71 replicates were done, being 29 contaminated and 42 without any contamination, corresponding to 41% and 59%, respectively (Figure 18);

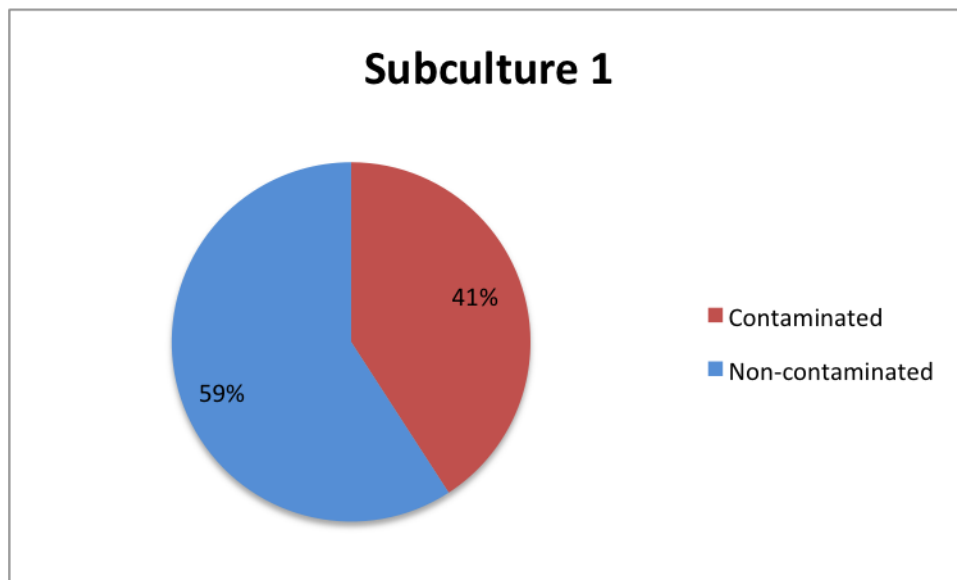


Figure 18. Contamination rate of the first subculture of *P. tinctorius*

- On a second main subculture, 18 replicates were done, being 4 contaminated and 14 without any contamination, corresponding to 22% and 78%, respectively (Figure 19);

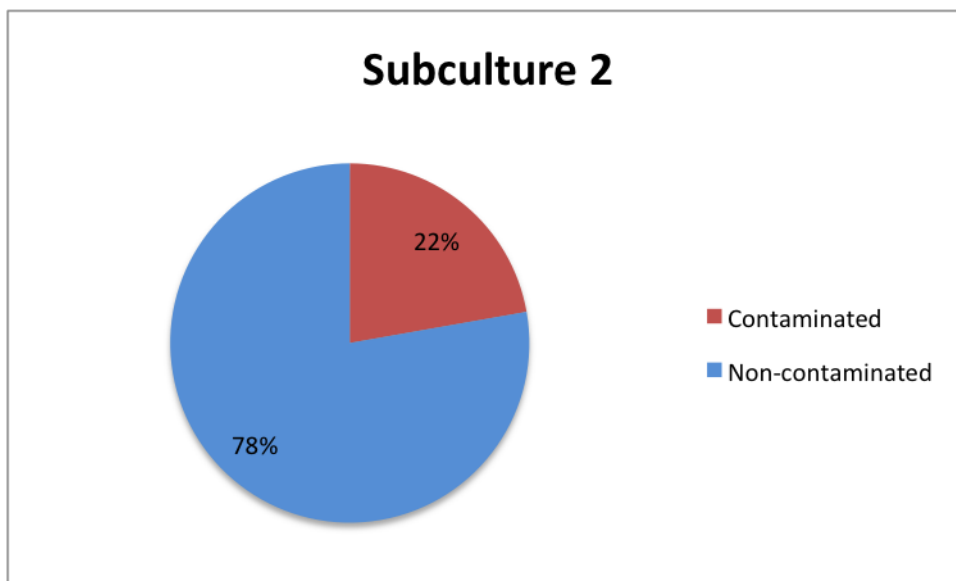


Figure 19. Contamination rate of the second subculture of *P. tinctorius*

- On a third main subculture, 41 replicates were done, being 15 contaminated and 26 without any contamination, corresponding to 37% and 63%, respectively (Figure 20).

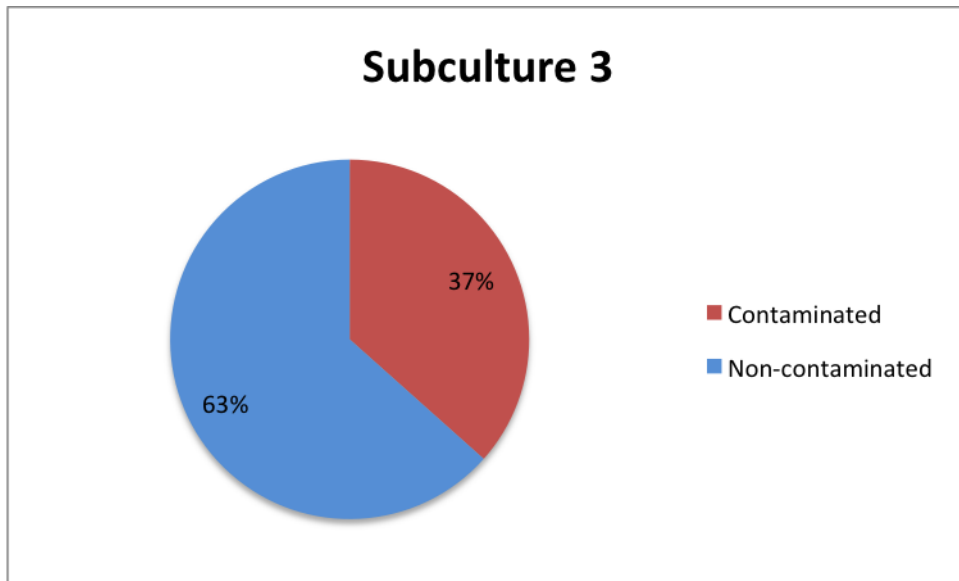


Figure 20. Contamination rate of the third subculture of *P. tinctorius*

- On a fourth main subculture, 36 replicates were done, being 2 contaminated and 34 without any contamination, corresponding to 6% and 94%, respectively (Figure 21).

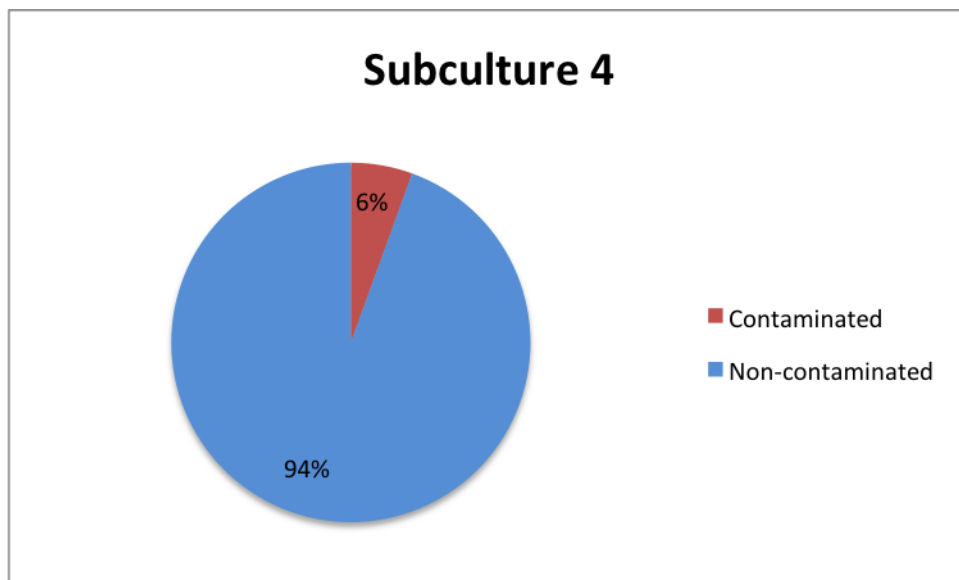


Figure 21. Contamination rate of the fourth subculture of *P. tinctorius*

3.4. Culture Media Colour Change

From the primary isolation and subsequent subcultures, it was possible to notice a colour change of the PDA medium, on the surroundings of the first isolated or subcultured mycelium. This colour varied according to the period of time in which the mycelium was incubated, changing from the normal light yellow colour of PDA, to strong yellow (2 to 10 days), to light brown (10 to 15 days) and dark brown or black in a final phase (15 days or more).

Although this change occurs on all petri dishes, the colour change was not constant, since the same number of days in incubation sometimes did not result in the same colour change of the medium, or the same intensity of the colour.

Figure 22 represents some examples of this colour change, with the associated incubation period.



Figure 22. Colour change of *P. tinctorius* cultures

It is possible to notice, on the Figure 22, that the colour may change significantly from the beginning to the end of the incubation period, showing a colour that with time becomes more dark and intense.

3.5. Choice of the Extraction Solvent

The amount of solvents, weight of the biomass, the dried amount recovered and the extraction yield are shown in the Tables 3 and 4, regarding *P. tinctorius* mycelium and fruiting bodies, respectively.

These results show that, regarding *P. tinctorius* mycelium, DCM-MeOH (2:1) had a dried amount recovered of 170.85 mg, corresponding to an extraction yield of 8.54%; EtOAc-MeOH had an extraction yield of 13.96%, with a dried amount recovered of 279.25 mg; DCM had the lowest extraction yield (6.89%), with 38.57 mg of dried amount recovered; MeOH had the highest extraction yield (45.9%), recovering 918,09 mg of dried amount.

In relation to the extractions made with *P. tinctorius* fruiting bodies, DCM-MeOH (2:1) had a dried amount recovered of 116.33 mg, corresponding to an extraction yield of 5.81%; EtOAc-MeOH had an extraction yield of 8.12%, with a dried amount recovered of 162.32 mg; just like on the extraction made with *Pisolithus tinctorius* mycelium, MeOH had the highest extraction yield, corresponding to 19.99% and 399.93 mg of dried amount recovered and DCM had the lowest extraction yield, with 3.71% and a dried amount recovered of 74.28 mg.

Table 3 – Solvents, total amount of solvents, weight of biomass, dried amount recovered and extraction yield of *P. tinctorius* mycelium.

Solvents	Total amount of solvents (ml)	Weight of biomass (g)	Dried amount recovered (mg)	Extraction yield (%)
DCM-MeOH (2:1)	75	2	170.85	8.54
EtOAc-MeOH (2:1)	75	2	279.25	13.96
MeOH	75	2	918.09	45.9
DCM	75	0,56	38.57	6.89

Table 4 – Solvents, total amount of solvents, weight of biomass, dried amount recovered and extraction yield of *P. tinctorius* fruiting bodies.

Solvents	Total amount of solvents (ml)	Weight of biomass (g)	Dried amount recovered (mg)	Extraction yield (%)
DCM-MeOH (2:1)	75	2	116.33	5.81
EtOAc-MeOH (2:1)	75	2	162.32	8.12
MeOH	75	2	399.93	19.99
DCM	75	2	74.28	3.71

The final crude extracts were submitted to a MTT assay, in order to evaluate their cytotoxic activity in HDCM, RKO, HEPG2 and T47D cell lines, analysing the viability of the cells at 24, 48 and 72 h after the exposure. Three different concentrations were tested simultaneously for each crude extract: 100 µg/ml, 10 µg/ml and 1 µg/ml.

The following data represents the effects of the crude extracts of *P. tinctorius* mycelium and fruiting bodies on the viability of T47D, HEPG2 and HCMEC/D3 cell lines (Figure 23, 24, 25, 26, 27 and 28).

3.6. *P. tinctorius* mycelium – cytotoxicity on cell lines

3.6.1. Cytotoxicity of the crude extracts on T47D cell line

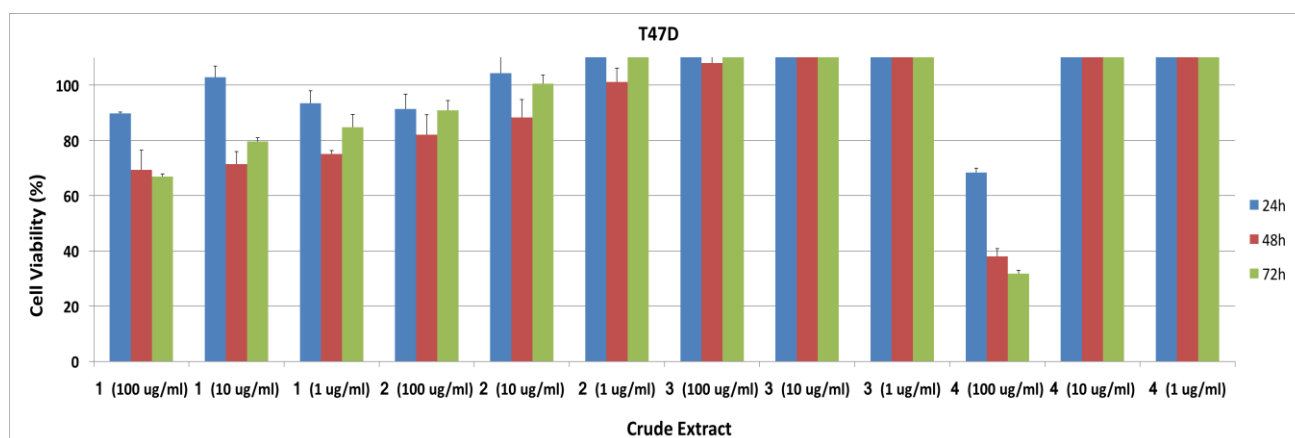


Figure 23. Effects of DCM-MeOH (2:1) (1), EtOAc-MeOH (2:1) (2), DCM (3) and MeOH (4) crude extracts at different concentrations and exposure times on the viability of T47D cell line. Values are presented as means $\pm$ SD analyses when compared with control group (n = 3).

- The extract obtained by DCM-MeOH (2:1) affected cell viability, with a slight reduction at all concentrations tested. After 72 h of exposure at 100 µg/ml, it presents the highest cytotoxicity, presenting about 65% of viable cells. After 48 h, the viability was below 75% for all tested concentrations.
- The extract obtained by EtOAc-MeOH (2:1) resulted in also a slight reduction in viability for all exposure times tested at the highest concentration. For the two lowest concentrations, 10 µg/ml and 1 µg/ml, the extract showed no reduction in cell viability.
- The extract obtained through MeOH extraction induced an increased cell viability at all exposure times and concentrations tested.
- The extract obtained using DCM showed a time dependent reduction in cell viability at 100 µg/ml. After 72 h of exposure, the cell viability value remained below 40% (31,8%). For the two lowest concentrations, no cytotoxicity was reported, with viability above 100%.

3.6.2. Cytotoxicity of the crude extracts on HEPG2 cell line

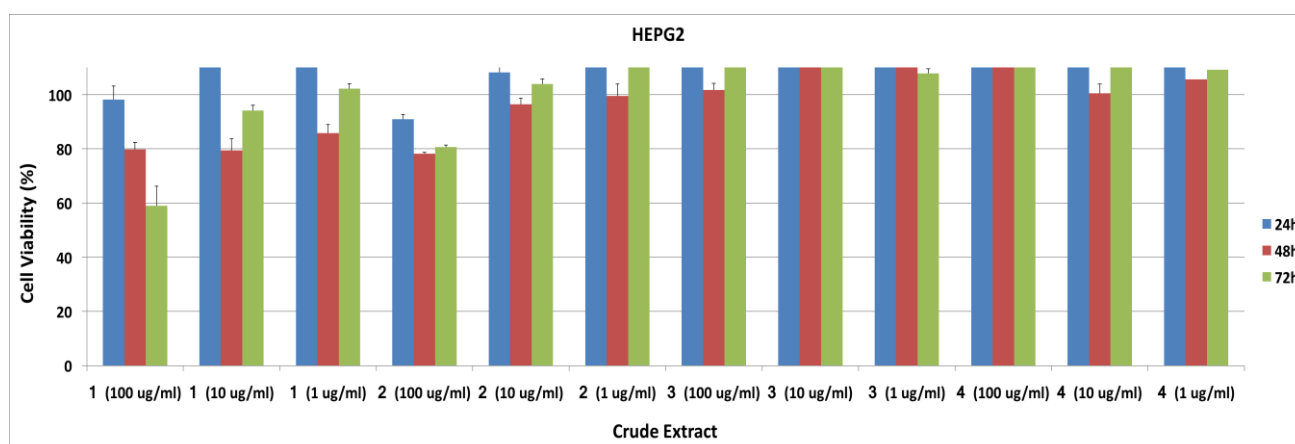


Figure 24. Effects of DCM-MeOH (2:1) (1), EtOAc-MeOH (2:1) (2), DCM (3) and MeOH (4) crude extracts at different concentrations and exposure times on the viability of HEPG2 cell line. Values are presented as means $\pm$ SD analyses when compared with control group (n = 3).

The crude extracts showed a reduction on the cell viability, but only with the extracts obtained with DCM-MeOH (2:1) and EtOAc-MeOH (2:1):

- The extract obtained using DCM-MeOH (2:1) showed a decrease in cell viability of the present line. At the concentration of 100 μ g/ml the recorded cell viability was about 80% after 48 h and less than 60% after 72 h, the lowest value being recorded in this line among all extracts and concentrations tested. A concentration of 10 μ g/ml demonstrated a reduction in viability after 48 h (less than 80%), which went up after 72 h. For the lowest concentration tested, cell viability was only reduced at 48 h exposure, to about 85%.
- Using EtOAc-MeOH (2:1) extract, cell viability was mostly affected at the concentration of 100 μ g/ml at all exposure times, being 80% or lower after 48 h of exposure. For the two lowest concentrations, viability was only reduced at 48 h, but without a substantial impact on its value.

3.6.3. Cytotoxicity of the crude extracts on HCMEC/D3 cell line

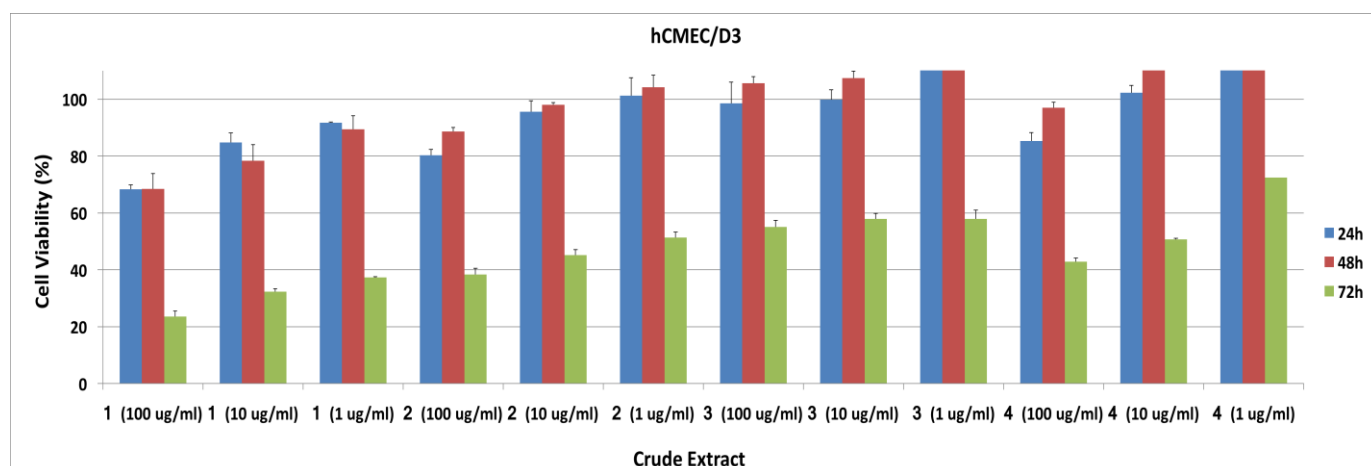


Figure 25. Effects of DCM-MeOH (2:1) (1), EtOAc-MeOH (2:1) (2), DCM (3) and MeOH (4) crude extracts at different concentrations and exposure times on the viability of HCMEC/D3 cell line. Values are presented as means $\pm$ SD analyses when compared with control group (n = 3).

The cell viability was always reduced after 72 h of exposure, for all tested solvents.

- The extract obtained by DCM-MeOH (2:1) showed a higher dose dependent cytotoxicity. After 72 h of exposure, viability was below 40%, at all tested concentrations. In relation to the 24 and 48 h period, it had a value of 68.3 and 68.2%, respectively, for the concentration of 100 μ g/ml, about 84.8% and 78.4% for the concentration of 10 μ g/ml and above 85% for 1 μ g/ml.
- The extract obtained using EtOAc-MeOH (2:1) demonstrated a viability value always less than 60% after 72 h, at all tested concentrations. After 24 and 48 h of exposure, the cell viability value was between 80 and 100%, being greater than 100% for the lowest concentration tested.
- The extract obtained with MeOH obtained a value of cellular viability that decreases only after 72 h of exposure, for all the concentrations tested, being between 50 and 60%.

- In relation to the extract obtained with DCM, cell viability demonstrated an increase with reduction of the concentration tested. After 24 and 48 h of exposure, cell viability was only reduced at the concentration of 100 µg/ml, ranging from 80 to 100%. After 72 h of exposure, values between 40 and 60% were obtained at concentrations of 100 µg/ml and 10 µg/ml and about 70% at the concentration of 1 µg/ml.

3.7. *P. tinctorius* fruiting bodies – cytotoxicity on cell lines

3.7.1. Cytotoxicity of the crude extracts on T47D cell line

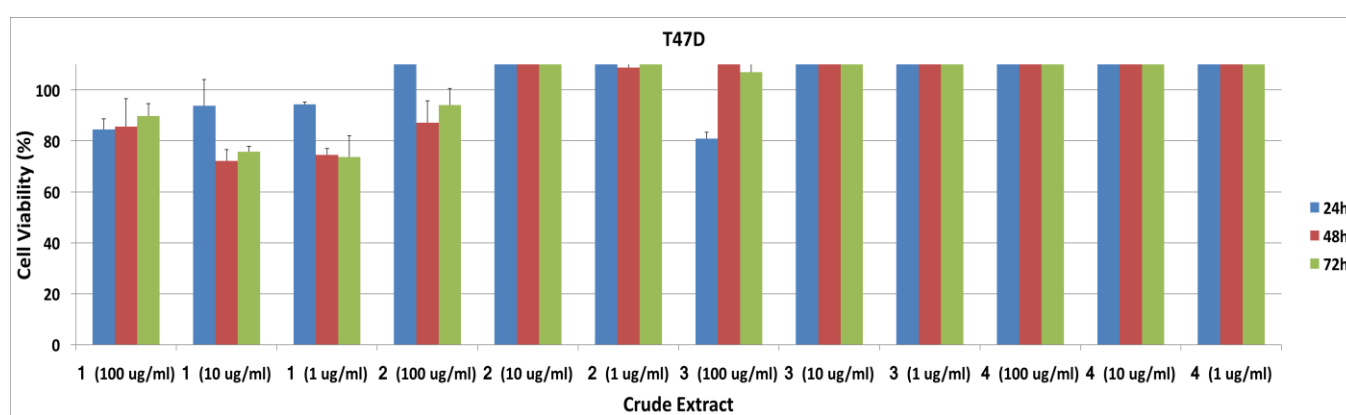


Figure 26. Effects of DCM-MeOH (2:1) (1), EtOAc-MeOH (2:1) (2), DCM (3) and MeOH (4) crude extracts at different concentrations and exposure times on the viability of T47D cell line. Values are presented as means $\pm$ SD analyses when compared with control group (n = 3).

The value of cell viability in this line was only reduced at all concentrations and times in the extract obtained by DCM-MeOH (2:1).

- Using the extract obtained with DCM-MeOH (2:1) at a concentration of 100 µg/ml, the cell viability was found to be between 80 and 90% at all tested exposure times; at 10 µg/ml and 1 µg/ml the viability was between 70 and 80% after 48 and 72 h of exposure.
- For the extract obtained by EtOAc-MeOH (2:1), the cell viability in this line was only affected at the concentration 100 µg/ml, but only after 48 and 72 h of exposure, with values between 80 and 100%.

- The extract obtained with MeOH showed cytotoxicity only at 24 h at the highest concentration, obtaining a cellular viability value of 80%.

3.7.2. Cytotoxicity of the crude extracts on HEPG2 cell line

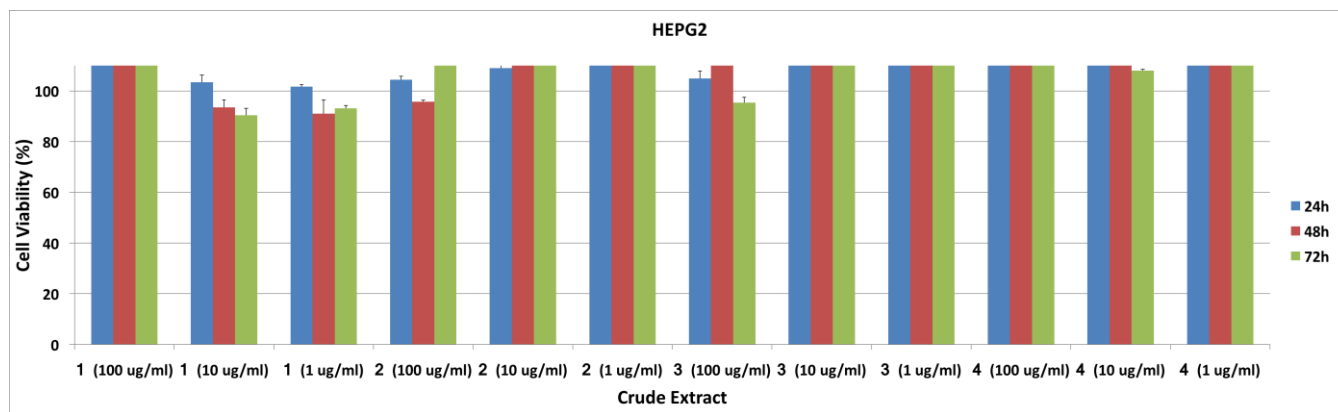


Figure 27. Effects of DCM-MeOH (2:1) (1), EtOAc-MeOH (2:1) (2), DCM (3) and MeOH (4) crude extracts at different concentrations and exposure times on the viability of HEPG2 cell line. Values are presented as means $\pm$ SD analyses when compared with control group (n = 3).

None of the extracts were found to induce cytotoxicity in the HEPG2 cell line. In this case only reductions around 10% were registered with the DCM-MeOH (2:1) extract at the concentration of 10 μ g/ml and 1 μ g/ml and in EtOAc-MeOH (2:1) and MeOH at 100 μ g/ml.

3.7.3. Cytotoxicity of the crude extracts on HCMEC/D3 cell line

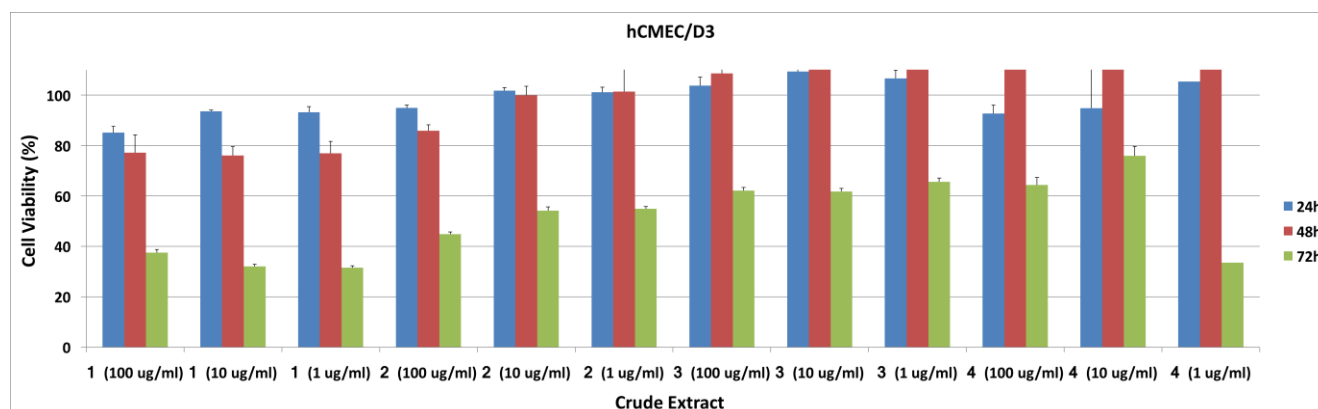


Figure 28. Effects of DCM-MeOH (2:1) (1), EtOAc-MeOH (2:1) (2), DCM (3) and MeOH (4) crude extracts at different concentrations and exposure times on the viability of HCMEC/D3 cell line. Values are presented as means $\pm$ SD analyses when compared with control group (n = 3).

The cell viability was always reduced after 72 hours of exposure, for all tested solvents.

- The extract obtained by DCM-MeOH (2:1) showed a higher cytotoxicity with increasing concentration. After 72 h of exposure, viability was below 40%, at all concentrations tested. In relation to the 24 and 48 h period, it had a value of between 75 to 90% for for all tested concentrations.
- The extract obtained using EtOAc-MeOH (2:1) demonstrated a viability value of about 60% or less after 72 h, at all tested concentrations. After 24 and 48 h of exposure, the cell viability was no affected.
- The extract obtained with MeOH obtained a value of cellular viability that decreases only after 72 h of exposure, for all the concentrations tested, being between 60 to 65%.
- In relation to the extract obtained with DCM, cell viability demonstrated strong variations, with no reduction at 48 h of exposure. At concentrations of 100 μ g/ml and 10 μ g/ml, the viability was about 90% at 24 h exposure,

and from 60 to 80% at 72 h of exposure. For the lowest concentration, the cell viability was only reduced to about 35% at 72 h.

3.8. Final extraction, fractionation and cytotoxic activity of the fractions

The final extraction was performed using DCM-MeOH (2:1) with 25.17 g of mycelium, previously lyophilised, and presented a final weight of 663.6 mg after the extraction, corresponding to an extraction yield of 2.65%.

After transferring the mixtures from the flasks to previously weighted vials and the solvents were evaporated on a rotatory evaporator with reduced pressure, the fractions biomass showed the weights presented in Table 5.

Table 5 – Weight of the fractionated biomass.

Fraction	Solvent Mixture	Dried Mass (mg)
A	10% EtOAc	40.22
	90% Hexane	
B	30% EtOAc	5.1
	70% Hexane	
C	40% EtOAc	51.89
	60% Hexane	
D	50% EtOAc	6.35
	50% Hexane	
E	60% EtOAc	1.91
	40% Hexane	
F	100% EtOAc	2.09
G	90% EtOAc;	13.35
	10% MeOH	
H	75% EtOAc	103.24
	25% MeOH	
I	100% MeOH	514.09
TOTAL		738.24

The nine fractions (A-I) were then submitted to a MTT test in RKO, T47D, HEPG2 and HCMEC/D3 cell lines at a concentration of 100 µg/ml, 10 µg/ml and 1 µg/ml, at 24, 48 and 72 h (Figures 29, 30, 31 and 32).

The following figures show the results of the cytotoxicity assay done for the nine fractions in RKO (Figure 29), T47D (Figure 30), HEPG2 (Figure 31) and HCMEC/D3 (Figure 32) cells.

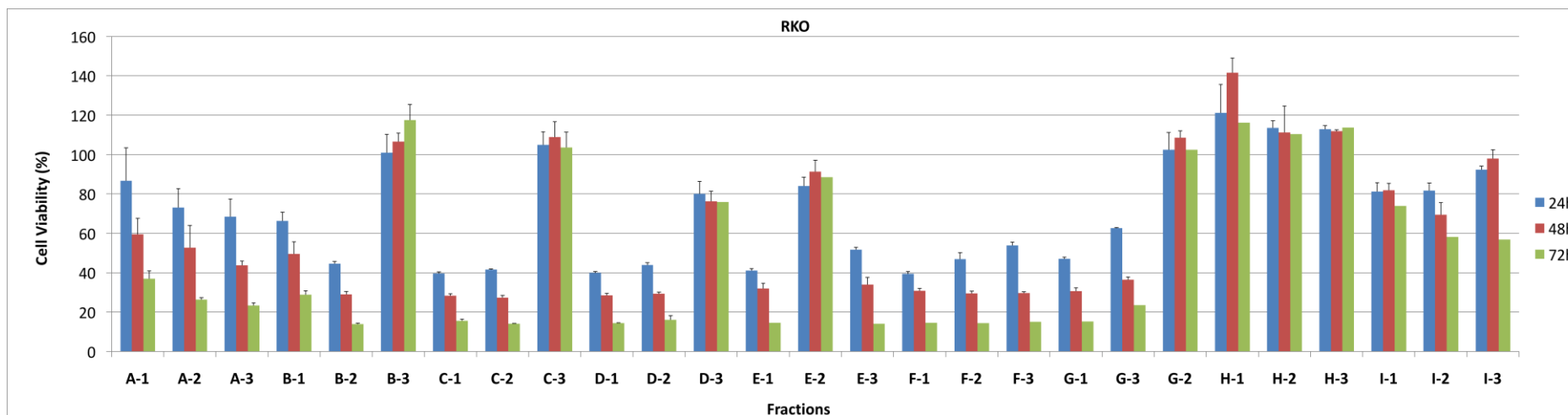


Figure 29. Effects of the fractions at different concentrations - 100 µg/ml (1), 10 µg/ml (2) and 1 µg/ml (3) - and exposure times on the viability of RKO cell line. Values are presented as means $\pm$ SD analyses when compared with control group (n = 3).

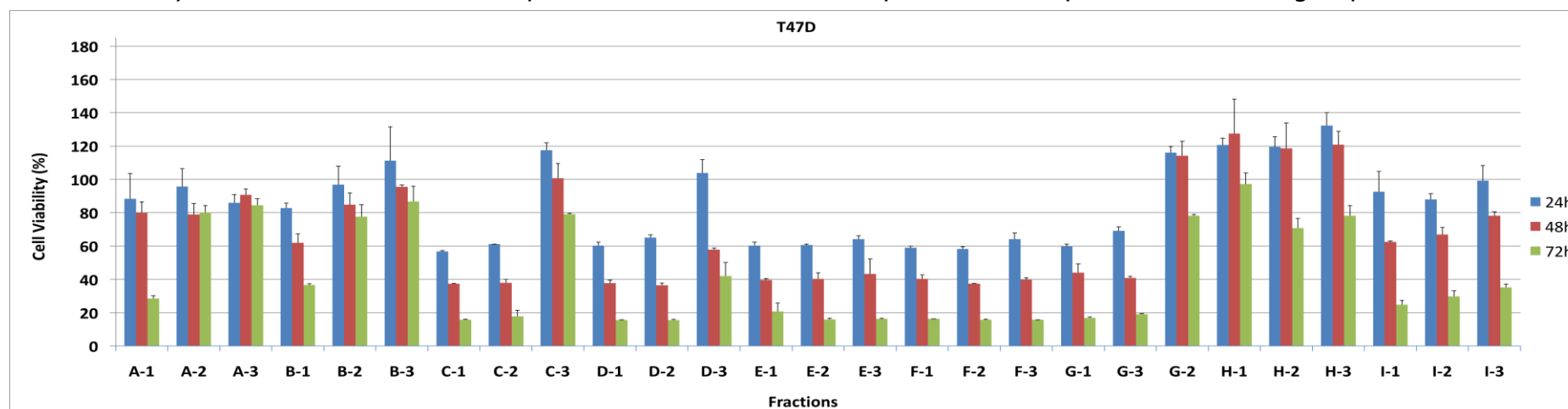


Figure 30. Effects of the fractions at different concentrations - 100 µg/ml (1), 10 µg/ml (2) and 1 µg/ml (3) - and exposure times on the viability of T47D cell line. Values are presented as means $\pm$ SD analyses when compared with control group (n = 3).

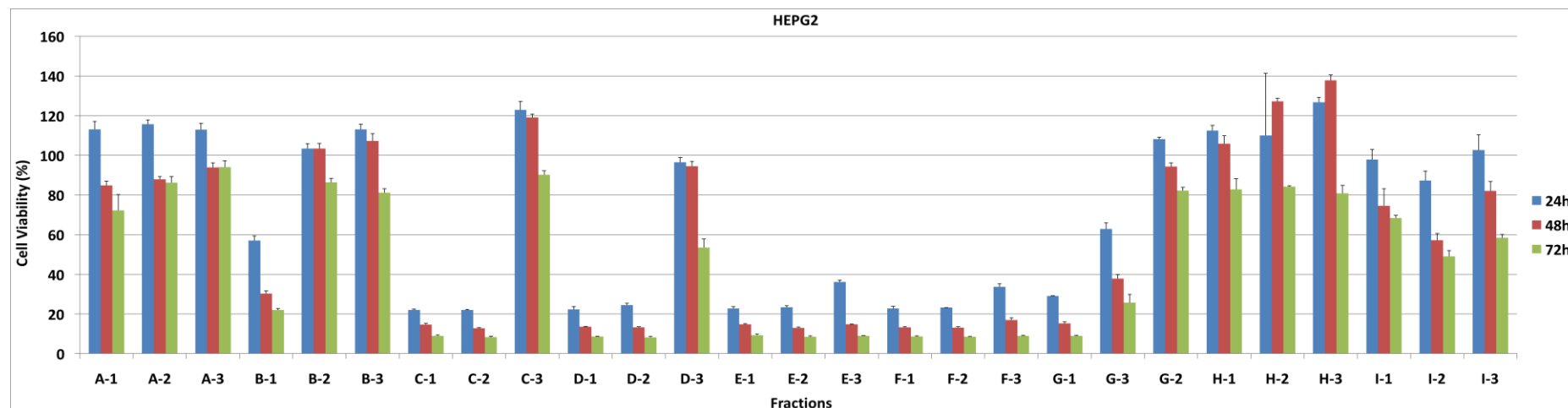


Figure 31. Effects of the fractions at different concentrations - 100 $\mu\text{g/ml}$ (1), 10 $\mu\text{g/ml}$ (2) and 1 $\mu\text{g/ml}$ (3) - and exposure times on the viability of HEPG2 cell line. Values are presented as means $\pm$ SD analyses when compared with control group ($n = 3$).

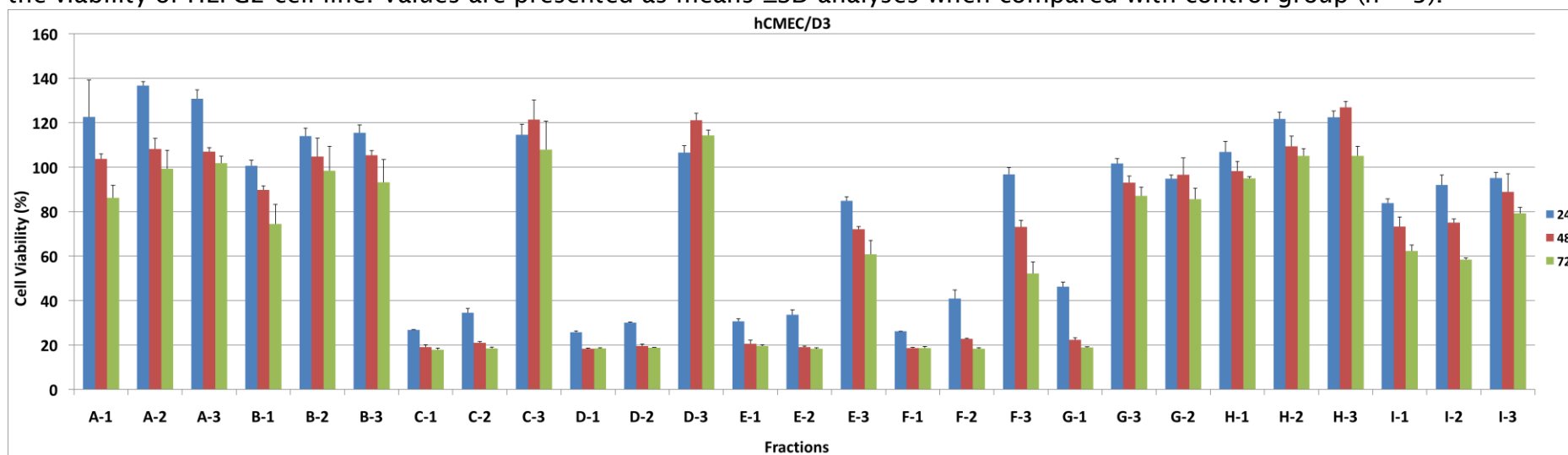


Figure 32. Effects of the fractions at different concentrations - 100 $\mu\text{g/ml}$ (1), 10 $\mu\text{g/ml}$ (2) and 1 $\mu\text{g/ml}$ (3) - and exposure times on the viability of HCMEC/D3 cell line. Values are presented as means $\pm$ SD analyses when compared with control group ($n = 3$).

3.8.1. Cytotoxicity of the fractions on the RKO Cell line

Regarding RKO cells, all fractions showed cytotoxicity, except the H fraction, which actually increased the cell viability:

- The fraction A presented a reduction of the cellular viability with the increase of the exposure time, also presenting a lower viability at a lower concentration. After 48 h of exposure the cell viability was always less than 60%, and after 72 h less than 40%.
- Fraction B showed lower cell viability at a 100 µg/ml concentration compared to the concentration of 10 µg/ml and at the lowest concentration tested no anticancer activity was observed. As in fraction A, at 48 h the cell viability was less than 50% for 100 µg/ml and 10 µg/ml, and for 72 h the value was less than 20% for the 10 µg/ml concentration.
- The C fraction, such as B, did not show cytotoxicity at the lowest concentration, which was less than 50% at 48 h and at 20% after 72 h of exposure.
- The D fraction showed cytotoxicity at all tested concentrations, decreasing the anticancer activity with the reduction of the concentration tested. There was a decrease in cell viability with increased exposure time at all tested concentrations. The cytotoxicity is lower in the concentration of 1 µg/ml compared to the obtained with 100 µg/ml and 10 µg/ml. In these, a cellular viability of less than 50% was observed after 24 h of exposure, and below 20% after 72 h of exposure.
- The E fraction showed cytotoxicity at all concentrations tested, with a lower anticancer activity at 10 µg/ml. After 24 h of exposure the higher concentration presented less activity, and the same occurred at 48 h. After 72 h the concentration with the highest reduction in cell viability was 1 µg/ml, although with approximate values at the tested concentration of 100 µg/ml.

After 48 h a cell viability of less than 40% was found for 100 µg/ml and 1 µg/ml and at 72 h was below 20% for the same concentrations.

- F fraction showed cytotoxicity at all concentrations, always below 60%. After 48 h of exposure, values below 35% were obtained, and after 72 h below 20%, at all concentrations tested. It is important to highlight that, after 72 h, all concentrations showed similar cell viability and that over time the viability always decreased.
- G fraction has no anticancer activity at the lowest concentration. However, at 100 µg/ml and 10 µg/ml the viability decreased over time, with values below 40% at 48 h of exposure and less than 25% at 72 h.
- Fraction I showed anticancer activity, although always with cellular viability higher than 55%, irrespectively of the concentration and the time of exposure. After 72 h of exposure at a concentration of 1 µg/ml, the highest activity was obtained in this fraction, corresponding to about 55% viable cells.

3.8.2. Cytotoxicity of the fractions on the T47D Cell line

In relation to this cell line, all the fractions presented cytotoxicity.

- The fraction A, although having demonstrated cytotoxicity in the cells at all concentrations, showed a significantly higher reduction at a concentration of 100 µg/ml after 72 h of exposure, which obtained a value below 30% of cellular viability.
- In fraction B, the anticancer activity increases over time and decreases with the reduction in concentration. At a concentration of 100 µg/ml, showed less than 40% after 72 h of exposure.
- The fraction C presented an activity that increased with the time of exposure and that decreased with the reduction of the concentration.

At the concentration of 100 µg/ml and 10 µg/ml, it presented values below 40% after 48 h exposure and less than 20% after 72 h.

- In fraction D, as in previous fractions, cytotoxicity increased with time. Concentrations of 100 µg/ml and 10 µg/ml had cell viability values below 40% after 48 h of exposure and less than 20% after 72 h. For the lowest concentration, cell viability remained below 60% after 48 h and 45% after 72 h.
- The fraction E presented a marked reduction of cellular viability for all concentrations tested. After 24 h of exposure, demonstrated a cell viability of about 60% and after 48 h this value reduces to about 40%. After 72 h, it presented values below 20% for both lower concentrations (10 µg/ml and 1 µg/ml).
- The fraction F, such as fraction E, presented values of cellular viability that decreased with the passage of time. After 48 h, showed a value lower than 40% for all concentrations, and at 72 h this value remained below 20%, being very similar in all tested concentrations.
- In the G fraction there was an increase of the cytotoxicity with the passage of time, and the two highest concentrations had a considerable higher anticancer activity. Thus, values below 20% after 72 hours exposure to the concentrations of 100 µg/ml and 10 µg/ml are emphasized.
- In fraction H, viability was only reduced after 72 h of exposure, presenting values between 70% and 80% for the two lowest concentrations.
- The fraction I presented an increase of the cytotoxicity with the increase of the time of exposure and a reduction of the anticancer activity with the reduction of the concentration, after 48 and 72 h of exposure. It should be noted that this fraction had values below 40% of cell viability for all concentrations after 72 h.

3.8.3. Cytotoxicity of the fractions on the HEPG2 Cell line

All fractions result in a reduction in cell viability in this line:

- Fraction A showed no cytotoxicity at any of the concentrations at 24 hours, presenting values of about 80% to 95% cell viability at 48 h, with the exception for the 72 h exposure to the highest concentration at which the value decreased to about 70%.
- Fraction B showed a marked cytotoxicity at the concentration of 100 µg/ml, compared with the two lower concentrations. At the highest concentration, it presents a cellular viability between 55 to 60% at 24 hours of exposure, less than 30% at 48 h and about 20% at 72 h. At the two lowest concentrations, viability was only reduced at 72 h, ranging from 80 to 90%.
- The C fraction shows a very high cytotoxicity, the effect being very similar to the concentration of 100 µg/ml and 10 µg/ml, with about 20% viability at 24 h, and below 20% at 48 h, reaching lower values of 10% at 72 hours of exposure. At the lower concentration, it presents reduction in cell viability only after 72 hours, with about 90% of viable cells.
- The D fraction showed a very low cellular viability at the concentration of 100 µg/ml and 10 µg/ml, with values between 20 and 25% at 24 h of exposure, and values below 15% after 48 and 72 h of exposure, for the same concentrations. At a concentration of 1 µg/ml, there was a slight reduction in the number of viable cells at 24 and 48 h, of about 95%, and after 72 h the value decreased to about 55%.
- Fractions E and F resulted in a very high cytotoxicity at all tested concentrations and exposure times. At 24 h they always present cellular viability values between 20 and 40%, and after 48 h the values were always lower than 20% and at 72 h about 10% or less, at all concentrations.

- Using the G fraction, the higher the concentration, the higher the reduction of cell viability. At the concentration of 100 µg/ml there were cellular viability values lower than 30% for 24 h, less than 20% for 48 h and less than 10% for the 72 h of exposure. This value rose at the two lowest concentrations, at 10 µg/ml between 60 and 65% for 24 hours, and between 20 and 40% for the two highest exposure times. At the concentration of 1 µg/ml, there was no reduction in viability at 24 h of exposure, revealing values between 80 and 95% for 48 and 72 h of exposure.
- The H fraction showed cytotoxicity only after 72 h of exposure for all concentrations, with values between 80 and 85% of cell viability.
- Fraction I demonstrated some anticancer activity in the present cell line, with viability values between 60 to 80% after 48 and 72 h of exposure at 100 µg/ml, values below 60% after the same exposure time at the concentration of 10 µg/ml and also less than 60% at the lowest concentration after 72 h.

3.8.4. Cytotoxicity of the fractions on the HCMEC/D3 Cell line

In most cases it has been shown that the lower the concentration, the lower the cell viability in the present cell line.

- In fraction A, cell viability showed a reduction at 72 h of exposure at the highest concentration, to about 85%, presenting values higher than 100% at the other exposure times tested.
- Fraction B showed cytotoxicity at a concentration of 100 µg/ml after 48 and 72 h of exposure, with values between 70 and 90% of cell viability. At the two lowest concentrations, there is a slight reduction in cell viability, from about 95% to 100%.
- Fractions C and D showed no cytotoxicity at the lowest concentration at any of the tested exposure times. However, at a concentration of

100 µg/ml and 10 µg/ml, it presented values between 20-40% at 24 h, and about 20% at 48 and 72 h.

- Using the fractions E and F, it was possible to verify that at 48 and 72 h of exposure, the viability value was about 20% in both fractions, for the two highest concentrations. At the concentration of 1 µg/ml the value was higher for both fractions, ranging from 80 to 100% after 24 h exposure, between 60 and 80% after 48 hours and from 50 to about 60% after 72 h of exposure.
- Concerning fraction G, the concentration of 100 µg/ml presented a much lower cellular viability when compared to the lower concentrations. Thus, it had a value of about 45% at 24 h of exposure and about 20% after 48 and 72 h at the highest concentration. This value rose in the two lowest concentrations, being between 80 and 100%, at 48 and 72 h of exposure.
- The H fraction showed only a slight reduction in cell viability at the highest concentration tested, revealing a viability equal to or greater than 95% after 48 and 72 h of exposure at the concentration of 100 µg/ml.
- Fraction I showed cytotoxicity at all concentrations and times tested. After 24 h, the value was greater than 80%, equal to or greater than 70% after 48 h and was between about 60 to 80% at 72 h.

3.9. NMR

Followed by the MTT assay, the fractions C, D, E, F and G were selected and further analysed through NMR in order to provide information about possible compounds present in the fractionated samples. Results are presented in Figures 34, 35, 36, and 37.

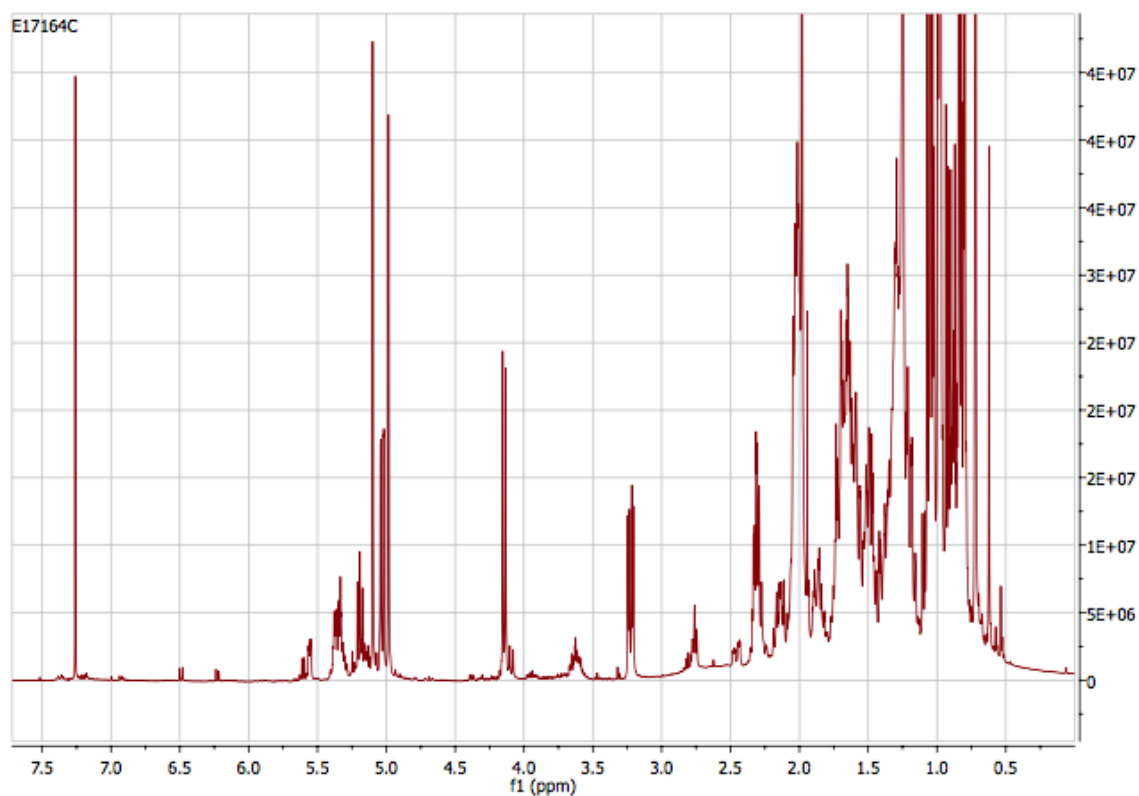


Figure 33. NMR spectrum of the C fraction

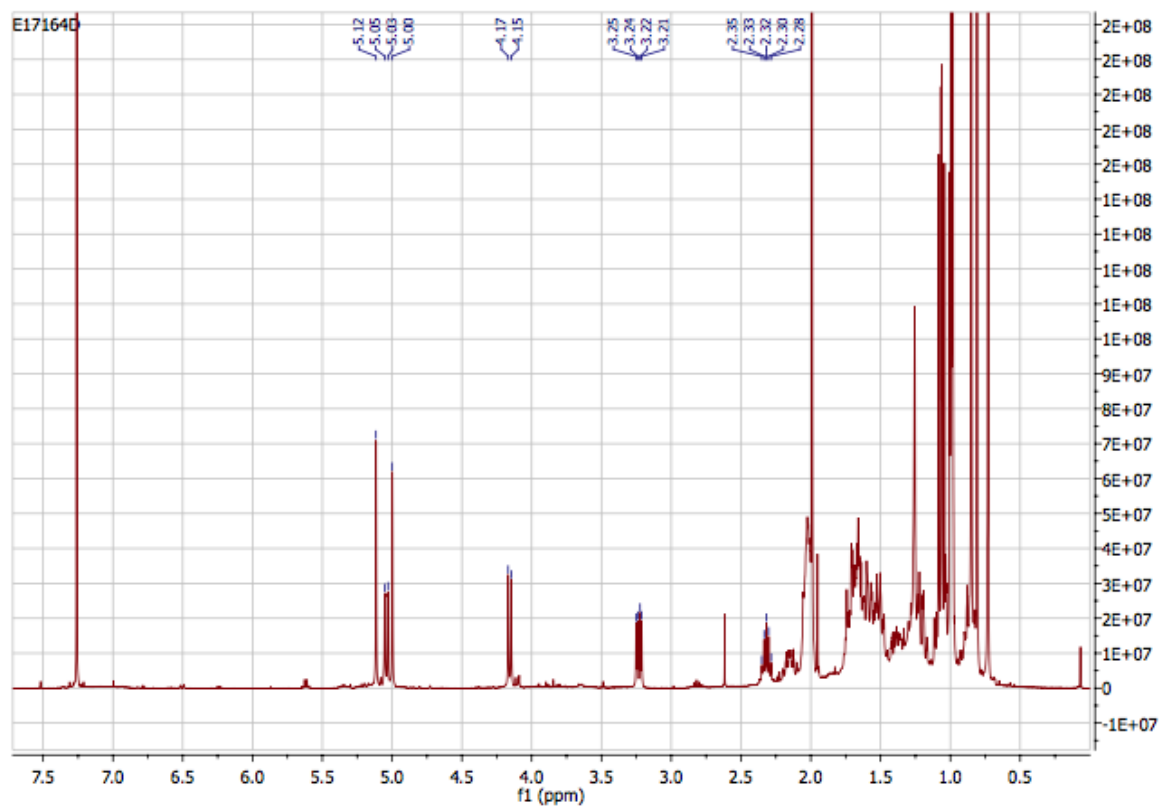


Figure 34. NMR spectrum of the D fraction

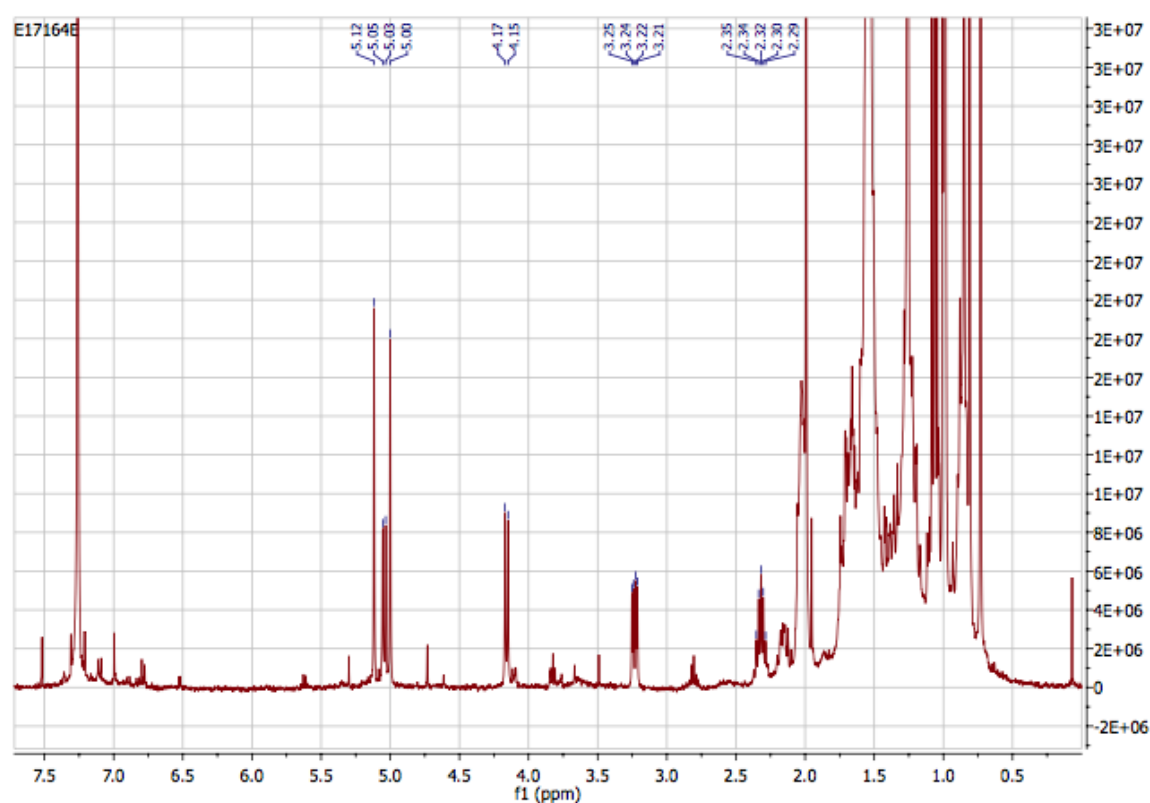


Figure 35. NMR spectrum of the E fraction

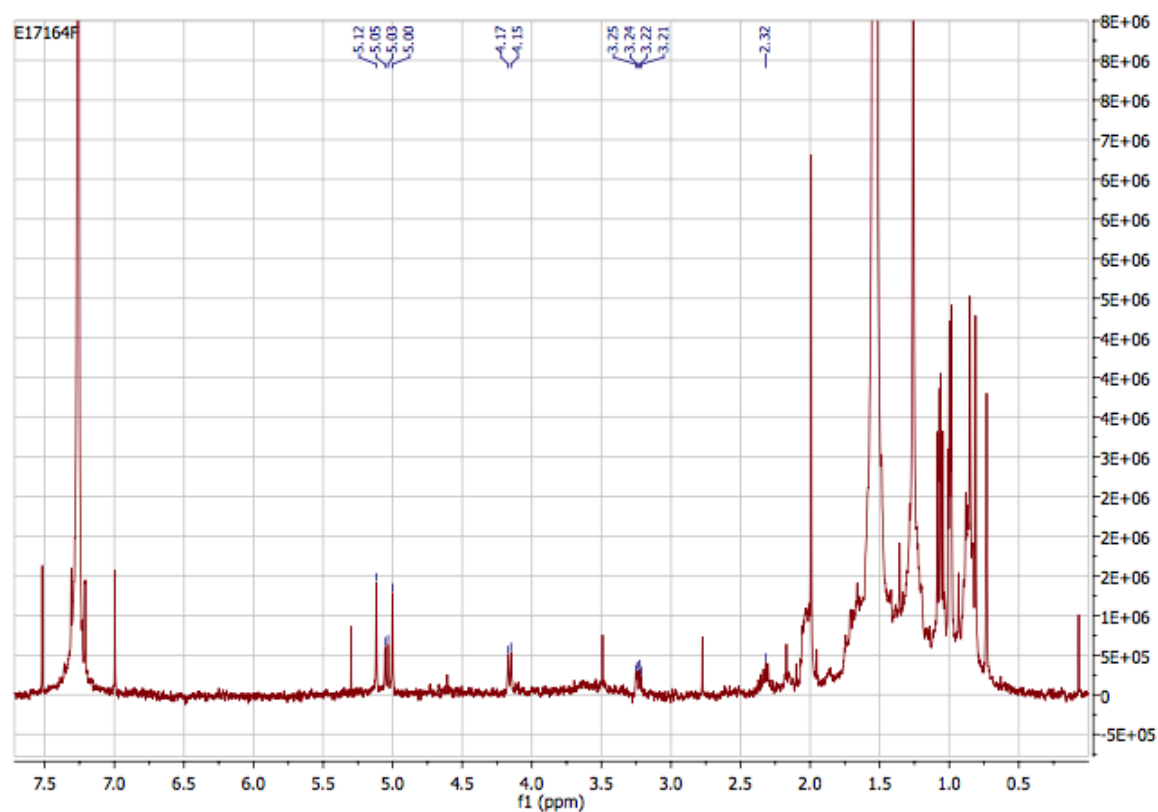


Figure 36. NMR spectrum of the F fraction

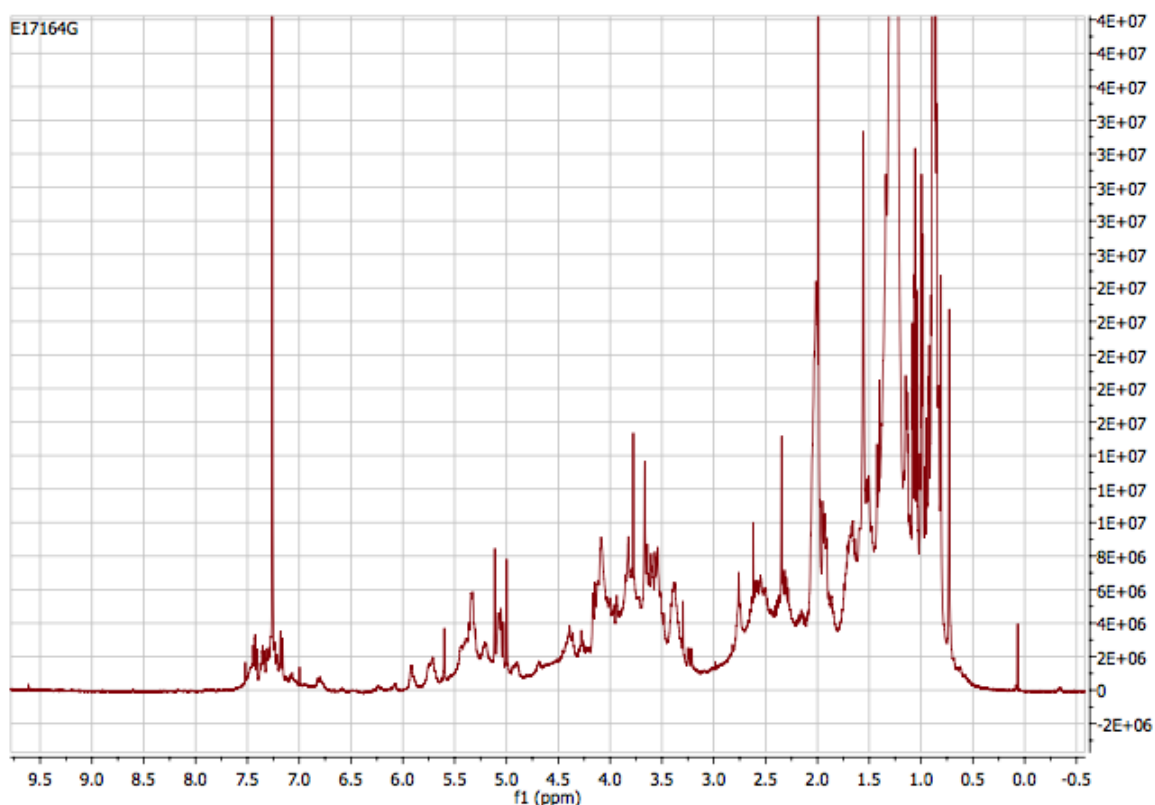


Figure 37. NMR spectrum of the G fraction

Analysing the profiles of the different fractions, it was possible to verify similarities among the different fractions, mainly in fraction D, E and F, that presented a set of equal peaks, marked in 5.12, 5.05, 5.03 and 5.00, 4.17 and 4.15 and 3.25, 3.24, 3.22 and 3.21. Thus, since all these fractions showed a very high cytotoxicity, a HPLC analysis was performed in an attempt to evaluate the fractions complexity and isolate the main components of the fraction.

3.10. HPLC

Fraction D (5.35 mg) was used with the objective of separate and isolate different compounds or mixtures of compounds. The following figure shows the general chromatography profile obtained through HPLC, and the following divisions in fractions (Figure 38).

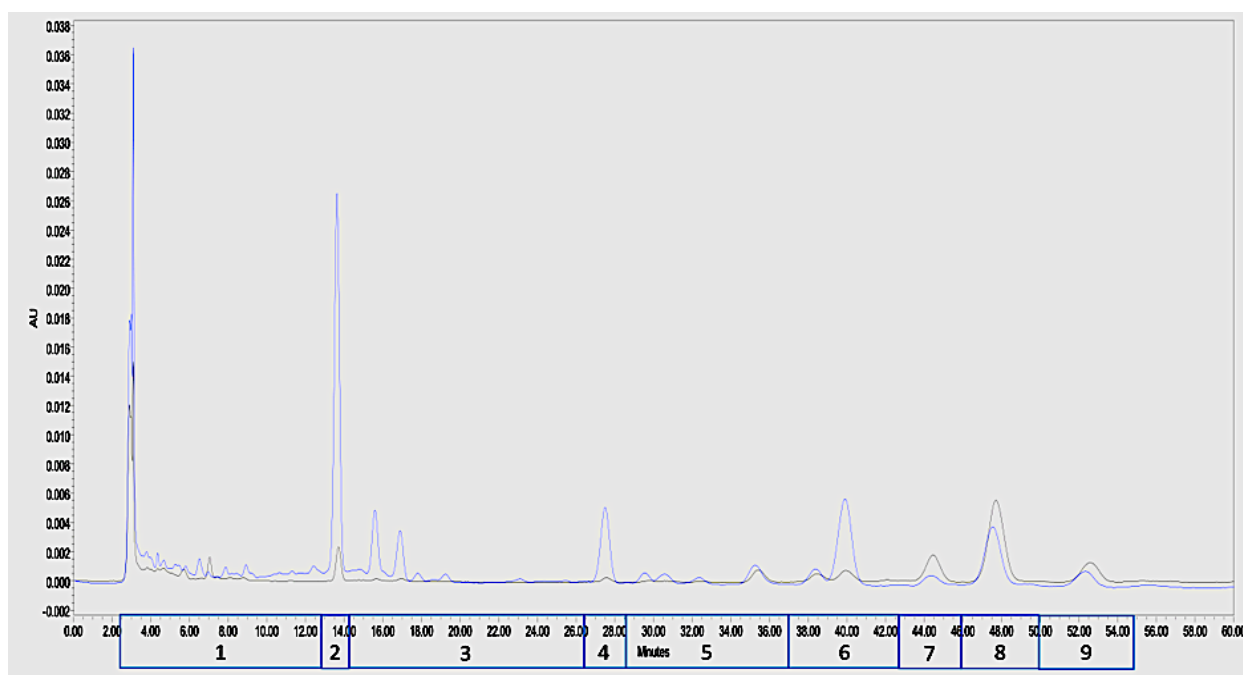


Figure 38. Chromatographic profile of the fraction D

After obtaining 9 different fractions (D1 to D9) 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 biomass showed the following weights, presented on the table 6.

Table 6 - Weight of the D subfractions biomass.

Fraction	Dried Mass (mg)
D1	3,54
D2	0,09
D3	0,14
D4	0,09
D5	0,02
D6	0,02
D7	0,03
D8	0,06
D9	0,12
TOTAL:	4,11

Given the low mass available in sub-fractions D1 to D9, no subsequent fractionation or purification was carried out.

3.11. Antimicrobial activity: Agar disk-diffusion method

The crude extracts of *Pisolithus tinctorius* mycelium and fruiting bodies, regarding the extractions made with Dichloromethane/Methanol (DCM/MeOH-2:1), Ethyl-acetate/Methanol (EtOAc/MeOH-2:1), Methanol (MeOH) and Dichloromethane (DCM), were submitted to a agar disk-diffusion method, and the antimicrobial activity was tested against 4 different bacteria strains: *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 25241) and the fungus *Candida albicans* (ATCC 10231). Figure 39 presents an example of the results after 24h incubation of the petri dish containing the bacteria/fungus and the disks with the correspondent extracts.

The antimicrobial potential is noticed when a halo with a determinable diameter is visible. Thus, none of the four crude extracts showed activity against any of the tested bacterial and fungal strains.

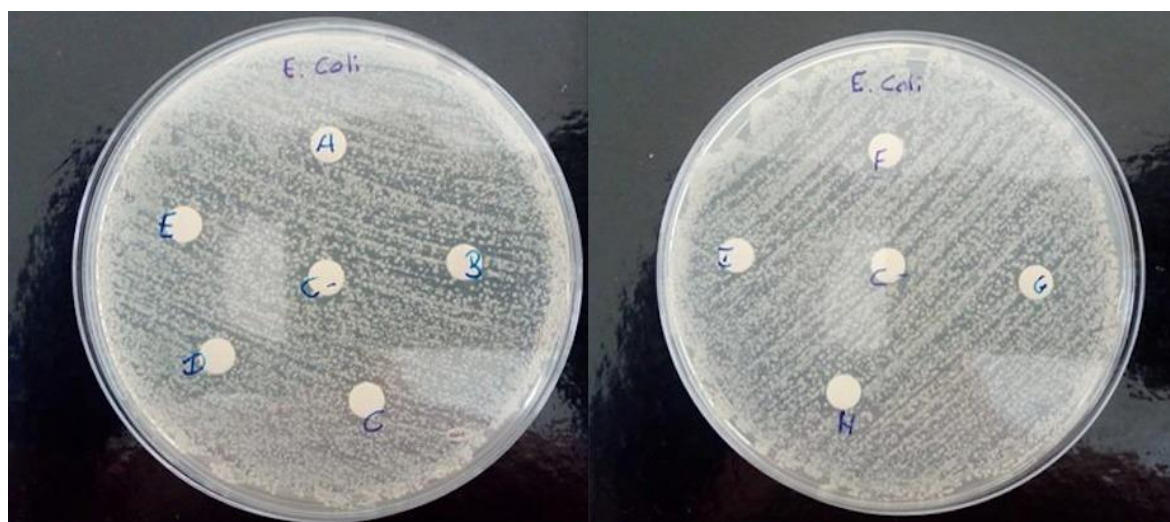


Figure 39. Agar Disk Diffusion test of the fractions in *Escherichia coli*

The extract fractions obtained by the VLC fractionation were also tested and, after testing the nine fractions (A to I) of *Pisolithus tinctorius* mycelium, it was possible to see that no antimicrobial activity was noticed.

4. Discussion

4.1. Isolation and Growth of the *P. tinctorius* mycelium

In order to evaluate the best medium to grow *P. tinctorius* mycelium, Malt Extract Agar medium (MEA), Sabouraud Dextrose Agar medium (SDA) and Potato Dextrose Agar medium (PDA) were tested.

The mean size of the mycelium was evaluated to determine which of the three media would result in the largest mycelial growth. The mycelium was measured taking into account its common circular shape, registering its diameter. The PDA medium showed a much higher growth than the other two media tested, averaging 45.59 mm after 20 days of incubation. These three nutritional media were chosen mainly because they are the most common and are widely used for fungal growth. The PDA medium showed the best efficiency, which was already expected given its wide use in fungi of this genus.

It should be noted that the mycelium did not grow at room temperature, and it was necessary to incubate at 25°C, as done by Coker & Couch (1928) and De Araujo et al. (2000), being also sealed with parafilm and incubated in the dark (Coker & Couch, 1928; De Araujo et al., 2000)

These same authors used the PDA as a culture medium, also testing other nutrient media, reporting that the PDA, as in the present work, would be the best and most suitable for *P. tinctorius*.

De Araújo et al. (2000) also measured the colonies after 15 days of incubation, obtaining a value of 23 mm (De Araujo et al., 2000). In the present study, the measurement performed after 20 days had a mean diameter of 45.59 mm, and growth was also evaluated every 5 days, obtaining mean values of 19.69 mm at the end of 5 days of incubation,

26.07 mm after 10 days, 37.23 mm after 15 days and 45.59 mm after 20 days. Comparing with the work developed by De Araújo et al. (2000) and analysing the growth after 15 days, the value obtained in the present study was 37.23 mm which, when compared to the value of 23 mm obtained by De Araújo et al. (2000), results in a difference of 14 mm. This difference in the growth of the mycelium can be justified by the difference of the collected specimen: as mentioned by different authors, *Pisolithus* can be distinguished by aspects such as morphological characteristics, symbiotic efficiency, ecology, physiology and geographic distribution (Anderson et al., 2001; Burgess et al., 1995; Cairney, 2002; Lamhamedi et al., 1990) and also, for example, given that *Pisolithus tinctorius* and *Pisolithus arhizus* are often considered the main species, this fact can demonstrate that despite the similarity of different specimens of the same species, these may contain slightly different biological and chemical characteristics (Bronchart et al., 1975; Coker & Couch, 1928; Watling, 1995). Additionally, Martin et al. (1988) reported that different host plants may be associated with different species of *Pisolithus* with different characteristics - which may also justify some difference in *Pisolithus* growth in the present work compared to that obtained by Araújo et al, since the specimens may have been obtained from different host plants, conferring different singularities (Martin et al., 1988).

The growth of *Pisolithus tinctorius* in petri dish was constant up to the 20th day, and by that time it began to decrease, probably due to the total consumption of its nutrients in the medium.

Along with the mycelium growth, the contamination rate was also evaluated. Thus, by counting contaminated cultures against the number of uncontaminated cultures, it was possible to verify that the most efficient medium for mycelial growth was the PDA, which obtained 83% of uncontaminated cultures compared to only 17% of contaminated cultures.

In an attempt to avoid contamination, the influence of penicillin-streptomycin on mycelial cultures was tested. It was possible to note that of the cultures with pen-strep, 53% were contaminated - that is, a greater amount of cultures contaminated than the uncontaminated ones. In contrast, of the isolations performed without pen-strep, only 17% of the petri dishes showed some

contamination, which reveals a higher efficiency and a lower rate of contamination of the medium without the antibiotic. Contaminations were mostly carried out by yeasts and filamentous fungi, leading to the likelihood of the antibiotic eliminating the bacteria, ending the competition for the nutrients of the medium and opening space for the growth and proliferation of a greater quantity of fungi and yeasts.

Regarding the subcultures of *P. tinctorius*, it was possible to observe that the contaminations were decreasing in successive subcultures, with the exception of the third of the four: in the first one, 41% of contaminated petri plates were recorded, the second 22% and in the fourth only 6%. This decrease in the contamination rate can be justified by the increasing purity of the subcultured mycelium, successively eliminating any impurities that may have come from the primary isolation. It should be emphasised, however, that contaminations can also arise from the handling of the materials or even from failures in the disinfection process, which may justify the exception obtained in one of the carried out subcultures or in some of the contamination rates obtained.

4.2. Culture Media Colour Change

During the incubation time of the mycelium, it was possible to notice a successive change in the colour of the culture medium. This colour varied from the normal colour of the medium (light yellow), to darker yellow from 2 to 10 days of incubation, to light brown (10 to 15 days) and dark brown or black after 15 days of incubation. Making the bridge with the study carried out by Gill et al. (1985), this colour change could be associated with the yellow pigmentation from the naphthalenoid pulvinic acid, present in high concentrations in *P. tinctorius* (Gill et al., 1985). This can also be reinforced by the fact that *P. tinctorius* is considered a natural dye, also used to dye tissues (Bessette & Bessette, 2001). Also taking into account the origin of the word "tinctorius", it is also possible to relate its meaning to its capacity: "tinctorius" originates from the word "tingō", which means "soak in dye", being an adjective for "of or pertaining to dyeing" - which suggests the presence by itself of an agent capable of changing the colour of the surrounding environment by contact.

It is important to emphasise that there are some exceptions in this colour change of the cultures, since some change the colour more quickly, probably due to the part of the fruit body that was isolated or subcultured.

Given the change in the colour of the medium, and because this change suggests the migration of one or more compounds into the medium, the agar was also extracted together with the mycelium.

4.3. Choice of the extraction solvent

Taking into account the yield of the extractions carried out with the four solvent mixtures or solvents - DCM-MeOH (2:1), EtOAc-MeOH (2:1), MeOH and DCM - it was possible to verify that the extraction yield was lower for DCM, followed by DCM-MeOH (2:1), EtOAc-MeOH (2:1) and MeOH, which obtained the highest yield, both in the extraction from the mycelium and in the extraction from the fruiting body. The extracts were also tested for cytotoxicity using cancer and normal cell lines by using the MTT assay.

MTT assay is a colourimetric assay to determine cell viability, in which the metabolically active cells establish a relation with the colour produced, allowing a count of the cell death and proliferation (Mosmann, 1983; van de Loosdrecht et al., 1994). The MTT reduction is influenced by the cellular metabolic activities caused by the NAD(P)H-dependent cellular enzymes - analysing the mitochondrial activity (Bahuguna et al., 2017; Berridge et al., 2005). MTT is the most common method used for evaluating cell viability and cytotoxicity of drugs (Berridge et al., 2005). This method is widely used in immunology, toxicology and cellular biology, being capable of evaluating the cytotoxicity of compounds by their ability to damage and destroy cells when a reduction decrease of MTT to formazan is noticed (Denizot & Lang, 1986; Mosmann, 1983; Sieuwerts et al., 1995). Some advantages of the MTT assay are the accuracy and reliability of this method, its fast execution and also the fact that is cheap (Denizot & Lang, 1986; Mosmann, 1983). Also, it is reproducible and HTS (High-throughput Screening) capable (Mueller et al., 2004). This method is also applicable in large-scale studies and tests, since it is performed in a 96 well plate and allows simultaneous test conditions, like

different concentrations and combinations – consuming also a small amount of cells and drugs, and read automatically (Hayon et al., 2003; Pieters et al., 1990). Thus, MTT proved to be an important tool on oncological research, in clinical practice to evaluate tumor cell sensitivity of individual patients and to preselect suitable chemotherapy, evaluating the potential efficacy of a single drug or a drug combination in cancer cell lines (Hayon et al., 2003).

It is possible to verify that the extract obtained with DCM-MeOH (2:1) was the only one that showed a reduction in viability in the three concentrations tested, in both cancer cell lines (T47D and HEPG2). On the other hand, all extracts affected the normal cell line (HCMEC/D3), depending on the concentration: the higher the concentration, the higher cytotoxicity reported. DCM-MeOH (2:1) was the one that demonstrated the higher cytotoxicity in this cell line as well.

The extracts obtained with the fruiting body, through different solvents, showed similar results: DCM-MeOH (2:1) reduces cell viability at the three different concentrations in T47D cells, being the only one of the four solvents tested. In HEPG2 cells there is slight cytotoxicity, but the extract obtained through this solvent was still the most effective. In normal cells (HCMEC/D3), it is the only one that reduced its viability at all tested concentrations and times of exposure - all extracts showed a high cytotoxicity at 72 h of exposure.

Comparing the extracts obtained through the mycelium and extracts obtained through the fruit body, DCM-MeOH (2:1) was the solvent that showed higher efficiency in the reduction of the viability of cancer cells and the extract obtained using the mycelium showed higher cytotoxicity, being transverse to both cell lines and affecting them at all tested concentrations.

Thus, since cytotoxicity is higher with DCM-MeOH (2:1), this was the solvent chosen, although it had lower extraction yields than other solvents such as EtOAc-MeOH (2:1) or MeOH, which showed low or no cytotoxicity - as in the study carried out by Alves et al. (2015), DCM-MeOH (2:1) was the solvent that showed the best cytotoxicity result in cancer cell lines when compared to solvents such as EtOAc-MeOH (2:1) and DCM, although in this study the

extraction was carried out from the spores of *Pisolithus tinctorius*. In addition, this extract demonstrated higher cytotoxicity when obtained from the mycelium than that obtained from the fruiting body - for this reason, and because there are no cytotoxicity studies in carcinogenic lines of extracts obtained from the mycelium isolated from the *Pisolithus tinctorius*, contrary to the fruiting body, the isolated mycelium was chosen for the follow-up of the work and for a final extraction and consequent fractionation.

Normal HCMEC/D3 cells were also affected: bridging the study performed by Montenegro et al. (2008), these same authors also tested a compound obtained through the *P. tinctorius* - the pisosterol - reporting inhibition in the tumor growth of Sarcoma S180 transplanted-mice, nevertheless affected normal cells of the kidney and liver, referring them as reversible, which may be the case for HCMEC/D3 cells in the present study. Furthermore, after isolating a compound and understanding its mechanisms of action, its use can be adapted and manipulated in order to minimise the side effects, perhaps by increasing its selectivity.

4.4. Final Extraction, Fractionation and cytotoxic activity of the fractions

Using 25.17 g of lyophilised biomass and 75 ml of solvent DCM-MeOH (2:1) an extraction yield of 2.65% was obtained, corresponding to 663.6 mg of final mass, which was less than the extraction previously performed with the same solvent. This can be explained because the ratio between biomass / solvent was higher, hence the loss of yield. That is, the protocol performed for the previous extraction was maintained and the amount of solvent used as well, but the amount of biomass used was substantially higher.

In the fractionation carried out by VLC, the masses obtained in the different fractions and after drying in the Rotatory evaporator system, revealed large differences, from a minimum weight of 1.91 mg (fraction E) to a maximum weight of 514.09 mg (fraction I). The substantially higher mass of the last fraction can be justified because the last step of the fractionation is the addition of 100% MeOH, where some silica is dissolved in this mobile phase and can incorporate the fraction and also, there is a high amount of relatively

polar compounds that are only dragged with a polar solvent like MeOH. Since the biomass before fractionation was 663.6 mg and the post-fractionation biomass was 738.24 mg, a yield of 100% could be considered because all the biomass was recovered after the VLC, even with the silica contamination, which may overestimate the recovered mass.

The fractions were then submitted to the MTT assay to evaluate their cytotoxicity in the cancer cells T47D, RKO and HEPG2 and in the normal HCMEC/D3 cell line. The present results revealed a high cytotoxicity of the fractions, especially fractions C, D, E, F and G, which substantially reduced the viability of the exposed cells. Of these fractions, the E and F fraction deserve special mention, since they present a very high and regular cytotoxicity, even at the lowest concentration (1 µg/ml), and after 72 h always resulted in less than 20% of cell viability of cancer cells - more specifically, fraction E had 14.2% viable RKO cells, 16.4% viable T47D cells and 9.0% viable HEPG2 cells after 72 h of exposure and the F fraction showed 15.0% viable RKO cells, 15.8% viable T47D cells and 9.0% viable HEPG2 cells, also after the same exposure time - which corresponded to a reduction of 83.6% to 91.0% when administered in cancer cell lines, even at a low concentration. The cell viability values were identical in E and F fractions, regardless of the concentration tested and therefore their cytotoxicity did not change significantly with the decrease in concentration. In addition to these two fractions, the above-mentioned fractions also obtained very particular results: after 72 h of exposure, fraction C showed a viability of 8.2% at 10 µg/ml concentration in HEPG2 cells, 14.10% at 10 µg/ml in RKO cells and 17.8% at 10 µg/ml in T47D; Fraction D presented a viability of 8.3% at 10 µg/ml concentration in HEPG2 cells, 14.5% at 100 µg/ml concentration in RKO cells and 15.6% at 100 µg/ml and 10 µg/ml in T47D and fraction G presented 9.0% at 100 µg/ml in HEPG2 cells, 15.3% at 100 µg/ml in RKO cells and 16.8% at 100 µg/ml in T47D. These values revealed a high efficiency of the extracts and point to the likelihood of some compound or mixture of anticancer compounds in their composition.

After isolation of the pisosterol triterpene, performed by Gill et al. (1989) this compound was the subject of several studies by Montenegro et al. (2004). These studies have demonstrated the ability of pisosterol to inhibit tumor

growth in 7 different cell lines (Montenegro et al., 2004), the ability to reduce viable HL-60 cells without affecting the normal PBMC (Montenegro et al., 2007) and their ability to inhibit C-MYC gene amplification, making it easier to combat cancer cells (Montenegro et al., 2004; Pereira et al., 2011). Considering these studies, and analysing the results of the present study, the hypothesis that *Pisolithus tinctorius* is able to produce compounds with anticancer activity is solid. This was also demonstrated by Alves et al. (2015), which proved the cytotoxicity of extracts - also fractionated - from *Pisolithus tinctorius* spores in cancerous MG63, T47D and RKO cells, with little effect on normal hCMEC/D3 cells. Thus, the present study also demonstrates that one or more compounds responsible for the highly elevated anticancer activity can be extracted from the purified mycelium of this fungus, being a pioneer study in the cytotoxicity tests using this structure of *P. tinctorius*.

Montenegro et al. (2004) and Burbano et al. (2009) tested the activity of pisosterol, extracted from *P. tinctorius*, comparing its activity with the activity of the drugs used in chemotherapy, such as doxorubicin and etoposide and pisosterol showed only a slightly lower potential. Thus, it can be emphasised that the potential compounds present in the fractions that demonstrated anticancer activity could be used in conjunction with these conventional drugs, reducing the side effects caused by them, and maybe making the treatment less aggressive and more effective.

4.5. NMR and HPLC: chromatography profile and subfractionation

Given the activity in the cancer cell lines, the fractions C, D, E, F and G were chosen for analyses by NMR and, after evaluation of the chromatographic profile of all these fractions, fraction D and E were chosen for a consequent fractionation by HPLC. These two fractions were selected for different reasons: for having similar NMR peaks, more available biomass, higher purity and cleanliness in the chromatographic profile and because both showed a high cytotoxic activity in the cancer cells.

The HPLC technique allowed subfractionation of the D fraction in 9 new fractions, aiming to separate the different compounds, obtaining pure substances. At the end, and starting with 5.35 mg, it was possible to recover

almost 100% of the extract used, although the subfractions obtained a much reduced mass, which prevented the characterization of the collected samples. However, as it is possible to verify a very strong anticancer activity in fraction C, it would be of interest to test in the future a purification of this same one also through subfractionation - fraction C had the largest available mass (51.89 mg) and corresponds exactly to a fraction obtained previously, according to polarity, to the fraction D1, which obtained the largest mass of the fractionation effected with the fraction D (D1 to D9). Thus, a more efficient purification can be achieved by using a more non-polar solvent mixture. However, it was possible to verify the existence of different compounds in the fraction D, visible in the chromatographic profile, and it will probably be possible to identify them accurately.

4.6. Antimicrobial Activity

Several authors have tested the antifungal and antibacterial activity of *Pisolithus* spp.: Kope and Fortin (1989) have shown that this species were able to produce metabolites on agar medium with antimicrobial capacity, and also demonstrated their activity against phytopathogenic fungi (Kope & Fortin, 1990). Tsantrizos et al. (1990) isolated and characterised the compounds named pisolithin A and pisolithin B - which reported antifungal activity (Tsantrizos et al., 1991), Shrestha et al. (2005) demonstrated the activity of *Pisolithus* extracts in inhibiting the growth of several bacteria (Vaidya et al., 2005), Ameri et al. (2011) demonstrated the activity of *Pisolithus albus* against different strains of *Staphylococcus aureus* (Ameri et al., 2011) and Mohan et al. (2015) have demonstrated the ability of *P. albus* to inhibit pathogenic fungi present in plants (Mohan et al., 2015).

In the present study, the crude extracts obtained initially from the mycelium and fruiting body using DCM-MeOH (2:1), EtOAc-MeOH (2:1), MeOH and DCM were tested against different bacteria strains and one fungus, in order to evaluate their antimicrobial effect when submitted to a disk diffusion agar test. This was done because the mycelium and the entire fruiting bodies may have different compositions and in addition, different solvents used for extractions can lead to a different amount of compounds and metabolites available in the composition of the final extract and thus, result in a different

way regarding the antimicrobial activity. After this test was carried out against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhimurium* and *Candida albicans*, no inhibition of the same was observed, and it was not possible to verify the existence of an inhibition halo and so, the crude extracts had no antimicrobial activity.

Also, the fractions obtained through VLC were tested against the same bacteria and fungus - because the same compounds that would be presenting cytotoxicity in the cancer cells could also have some antimicrobial effect when submitted to a disk diffusion agar test. However, no inhibition was observed and so, the fractional extracts had no identifiable antimicrobial activity.

The fact that several authors have reported *Pisolithus* antimicrobial activity and in the present work this potential has not been verified can be explained by different hypotheses. Ameri et al. (2011), Shrestha et al. (2005) and Mohan et al. (2015) used a different species in the tests performed: the *P. albus* (Ameri et al., 2011; Mohan et al., 2015; Vaidya et al., 2005). This may mean that, because a different species is used, the metabolites and substances generated may be different from those obtained from *P. tinctorius*; The method of extracting and obtaining the product to be tested was also different: Kope and Fortin (1989) used a liquid medium Mannitol Nitrate Motility Agar (MMN) for the growth and extraction of the extract, Tsantrizos et al. (1910) isolated Pisolithin A and Pisolithin B and tested the two isolated compounds, Ameri et al. (2011) obtained the test extract from the milled fruit body of *P. albus* and Mohan et al. (2015) used MMN as a culture medium to grow the different ectomycorrhizal (ECM) used (Ameri et al., 2011; Mohan et al., 2015). In the present study, in contrast, the extract was obtained by *P. tinctorius* previously isolated on a PDA medium, extracting the mycelium with different solvents, or the lyophilised and milled fruiting body, which can give rise to final extracts with different chemical compositions without antimicrobial activity.

It should be noted that Ameri et al. (2011) tested the *P. albus* collected from two different tree species, *Acacia auriculiformis* and *Eucalyptus globules* (Ameri et al., 2011). *P. albus* isolated from *Eucalyptus globules* showed

activity against MRSA and those obtained from *Acacia auriculiformis* did not show any inhibition. Thus, different isolates of *P. tinctorius* from different host plants may also result in distinct antimicrobial activities.

Although in the present study there was no antimicrobial activity reported of any extract tested, it would be of special importance in the future to test different culture media, different extraction solvents or to isolate *P. tinctorius* from different host plants, with the objective of understanding which compound or mixture of compounds are responsible for the antimicrobial activity, as it can maybe be used as a natural product in the fight against various bacteria and fungi, if extracted and isolated in a precise way.

5. Conclusion

Fungi, as in the case of metabolites isolated from mushrooms, have long been associated with beneficial medical properties and have already been tested as antioxidants, anti-inflammatories, antivirals or even antitumor agents (Smith et al., 2002).

With the purpose of aiding the fight against cancer and other pathologies, the study of the *P. tinctorius* seems very pertinent. Firstly, because of the scarcity of research focused on the potential of activity of its mycelium and secondly, given the myriad of biological and chemical potentials of this fungus. Cancer is a disease responsible for the deaths of millions of people per year, and this number is expected to have an increase in time, reaching about 22 million cases on the next decade. Thus, using this fungus on a medicinal level with the support of studies that can show activity in that area reinforces the window of opportunity present in this dissertation, making this a rather ambitious study.

The anti-cancer drugs currently available on the market demonstrate several aggressive side effects, in addition to the lack of selectivity, which may reflect the emerging need to find a new form of treatment that is both effective and less toxic to the patient's body (Leszczyniecka et al., 2001;

Patel & Goyal, 2012). Today, conventional cancer-related therapies involve surgeries, chemotherapy, and radiation therapy, depending on the type of cancer and its development in the patient, resulting in irreversible damage and increased patient fragility (Chan et al., 2009; Silva et al., 2012).

For this reason, the search of natural methods with less secondary effects has increased in the last years, leading to a special attention on the research regarding organisms like fungi, which already have been associated with anticarcinogenic activities in more than 50 species worldwide, with a particular detail: the high level of tolerance from the human body to their action and the non-harmful secondary effects on the organism (Kidd, 2000).

The present study showed high cytotoxicity values of *P. tinctorius* mycelium extracts in different types of cancer cell lines, proving its potential as a possible future resource of anticancer compounds. Nevertheless, it is necessary to study how the normal HCMEC/D3 cells may be affected, in order to try to cancel this fact, understanding the mechanism of action of these extracts. The future of this study will involve the isolation of one or more compounds that will be responsible for cytotoxicity in cancer cells, as well as the way these compounds act in these same cell lines.

In addition to the use of the mycelium and compounds of the *P. tinctorius* as a biologically active product with anticancer properties, this study has other potentialities in mind, such as its use as an antibacterial, antifungal, antioxidant or anti-inflammatory, based on the idea of a future creation of a drug that can conglomerate some of these capabilities.

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