**Universidade do Minho**
Escola de Ciências

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# Study of antitumor and immunomodulatory activities of wild mushroom extracts

Dissertação de Mestrado
Mestrado em Genética Molecular
Trabalho realizado sob orientação da
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Co-orientação da
**Prof. Doutora Maria Helena Vasconcelos**
Co-orientação institucional da
**Prof. Doutora Ana Arminda Lopes Preto Almeida**

Outubro 2014

Outubro 2014

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Study of antitumor and immunomodulatory activities of wild mushroom extracts

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**Ano de Conclusão**: 2014

**Designação de Mestrado:**

Mestrado em Genética Molecular

Part of the work carried out during this thesis has been included in the following publications:

**Paper submitted for publication:**

A. BIZARRO, I.C.F.R. Ferreira, M. Soković, L.J.L.D. van Griensven, D. Sousa, M. H. Vasconcelos and R. T. Lima (**Submitted**) "*Cordyceps militaris* (L.) Link fruiting body reduces the growth of a non-small cell lung cancer cell line by increasing cellular levels of p53 and p21"

**Poster communications:**

A. BIZARRO, I.C.F.R. Ferreira, M. Soković, L.J.L.D. van Griensven, M. H. Vasconcelos and R. T. Lima (**Submitted**) "*Cordyceps militaris* (L.) Link fruiting body reduces NCI-H460 cellular viability through a mechanism involving p53 and p21" Submitted to XX Encontro Luso-Galego de Química, to be held in 26 to 28 November 2014, Porto, Portugal.

A. BIZARRO, I.C.F.R. Ferreira, M. Soković, L.J.L.D. van Griensven, M. H. Vasconcelos and R. T. Lima (**2014**) "Effect of *Cordyceps militaris* methanolic extract in NCI-H460 tumor cells", IJUP 14 - 7th meeting Young Research of the University of Porto, 12 to 14 February 2014, Porto, Portugal. Presented by A. BIZARRO. (Annex I)

# ACKNOWLEDGMENTS

The completion of this project represents, not just another cycle of studies completed, but also the beginning of a new stage. More than just an experiment in the lab, this project was a great help in terms of personal growth. I see this section as a formality, since these words will not be enough to thank everyone properly, however I would like to express my gratitude to all those who, in some way, helped me in this journey.

First of all, I would like to express my deepest gratitude and appreciation to Dr. Raquel T. Lima, my supervisor, for planning this study, for all the help and useful assistance during the course of the study, for the guidance, support, encouragement and friendship. For the hours we spent on the phone and on the computer discussing ideas, for the advices, patience and experience, which were proved to be essential for the completion of this work. Once again Raquel, thank you so much!

I would like also to emphasize my deep gratefulness to Prof. Dr. Helena Vasconcelos, my co-supervisor, for having received me so gently in her group, for all the confidence that she always had in me and in my abilities, thus encouraging me to progress and grow as a future researcher and for her kind assistance and valuable advices.

I must also thank Prof. Ana Preto for the support and clarifications and Prof. Dr. Maria João Sousa, since it was due to her that I meet Prof. Dr. Helena Vasconcelos and I chose this project, which has been an adventure for me and that has increased my love for science.

I would like also to express my sincere thanks and gratitude to Diana Sousa, Filipa Reis and Vanessa Rodrigues, for valuable advices and continuous support during the course of this study.

I would also like to thank Prof Isabel Ferreira (for providing her expertise and the mushroom extracts used in this study) and Prof. Anabela Cordeiro da Silva, who had a crucial role in this work, for the helpful discussions regarding the immunomodulatory studies, for providing the THP-1 cell line for my experiences and for kindly offering me access to equipment necessary to this work.

I would like to thank also my lab mates of Department of Biological Sciences of Faculty of Pharmacy in Porto, Maria João Lima, Joana Maria Pereira, Maria Paula Reis, Joana Durão, Matilde Monjardino, João Botelho and Carla Lima for their friendship, motivation and for the sharing of knowledge and experiences. Thanks also to Cristina and Nuno for the help and support and for ensuring that we have always the necessary tools to develop a good work.

I would also like to thank to those that I carry always in my heart, Guilherme (thanks Mr.C ;)), André, Ju, Pedro, Catarina, Cris, Romão, Jorge, Helder, Vitor, Miguel, Carla, Gisa, my dance girls (and Luis) and my Master girls (and boys) for their friendship, support, encouragement and patience. For all the moments of relaxation and fun, essentially, for always being there when I needed it most, making it all worthwhile.

And last yet not least, to my parents and sister (love you Bea), to whom I dedicate this thesis, for the unconditional support, patience and love. For being present in all the important moments of my life and for making me the person that I am today.

# ABSTRACT

Mushrooms and their compounds are widely appreciated, not only for their nutritional but also for their medicinal properties. In fact, the search for various bioactive properties in different mushroom extracts or in the compounds isolated from those mushroom has been the focus of attention from the scientific community working in the area of natural products. The present work has focused on the study of the antitumor and immunomodulatory properties of extracts from three different mushrooms. Thus, the first aim of the present work was to gain insight into the mechanism of action of a methanolic extract of *Cordyceps militaris* in the non-small cell lung cancer cell line NCI-H460, since this extract had previously been shown to have tumour cell growth inhibitory activity in this cell line in particular. In addition, the second aim was to study the immunomodulatory activity of the *Suillus luteus* polysachararidic (PLS) extract and of the *Morchella esculenta* phenolic extract, using the monocytic THP-1 cell line which differentiates into machrophages upon stimulation.

The response of NCI-H460 cells to the methanolic extract of *C. militaris* was studied regarding its effect on cellular viability, proliferation, cell cycle profile, apoptosis and DNA damage. Results showed that treatment with the methanolic extract of *C. militaris* caused a decrease in NCI-H460 cellular proliferation, a cell cycle arrest at G0/G1 and an increase in apoptosis. Interestingly, treatment with the extract was shown to increase the cellular levels of p53 and p21. Moreover, this study also showed evidence of cellular DNA damage caused by this extract, since increased levels of P-H2A.X and 53BP1 foci/cell were observed. Overall, this part of the work suggested that the methanolic extract of *C. militaris* affected NCI-H460 cellular viability through a mechanism which involved DNA damage and p53 activation.

In addition, preliminary experiments were carried out to gain insight into the immunomodulatory potential of a PLS extract of *S. luteus* and of a phenolic extract of *M. esculenta* in THP-1 cells. Results showed that neither of the extracts presented cytotoxicity towards THP-1 cells or induced THP-1 monocytes differentiation into macrophages. Interestingly, the PLS extract of *S. luteus* caused a dose-dependent increase in the metabolic activity of THP-1 monocytes, probably due to increased proliferation. These preliminary results need be further confirmed and continued in future work.

Overall, the work carried out in this thesis further supports the potential of mushrooms extracts in the search for bioactive compounds.

# RESUMO

Os cogumelos e seus compostos são muito apreciados, não só pelas suas propriedades nutricionais, mas também pelas medicinais. De facto, a procura de propriedades bioativas em diferentes extratos de cogumelos ou em compostos isolados desses cogumelos, tem sido um foco de interesse da comunidade científica que trabalha na área de produtos naturais. O presente trabalho visou o estudo de propriedades antitumorais e imunomoduladoras de extratos de três cogumelos diferentes. Assim sendo, como primeiro objetivo deste trabalho pretendeu-se analisar o mecanismo de ação de um extrato metanólico de *Cordyceps militaris* na linha celular NCI-H460 (de cancro do pulmão de não pequenas células), uma vez que tinha sido previamente demonstrado que este extrato inibe o crescimento celular, destas células em particular. Além deste, um segundo objetivo visou o estudo da atividade imunomoduladora de um extrato polisacarídico (PLS) de *Suillus luteus* e de um fenólico de *Morchella esculenta*, usando a linha celular monocítica THP-1 que se diferencia em macrófagos, após estimulação.

Foi estudada a resposta das células NCI-H460 ao tratamento com o extrato metanólico de *C. militaris* relativamente ao efeito na viabilidade celular, proliferação, perfil do ciclo celular, apoptose e no dano no DNA. Os resultados demonstraram que o tratamento com o extrato metanólico de *C. militaris* nas células NCI-H460 diminuiu a proliferação celular, bloqueou o ciclo celular nas fases G0/G1 e induziu apoptose. De particular interesse foi o facto de este extrato causar um aumento dos níveis de p53 e p21. Para além disso, este estudo também mostrou ainda evidências de danos no DNA causados por este extrato, dada a observação de aumento quer nos níveis de P-H2A.X como no número de 53BP1 *foci*/célula. Em geral, esta parte do trabalho sugeriu que o extrato metanólico de *C. militaris* diminuiu a viabilidade celular das células NCI-H460 por um mecanismo que envolve a ativação de danos no DNA e de p53.

Neste trabalho, foram realizadas experiências preliminares para averiguar o potencial imunomodulador de um extrato PLS de *S. luteus* e de um extrato fenólico de *M. esculenta* em células THP-1.

Os resultados demonstraram que nenhum dos extratos analisados apresentou citotoxicidade para células THP-1 ou induziu a diferenciação desta linha celular de monócitos em macrófagos. De particular interesse foi o facto de se verificar que o extrato PLS do *S. luteus* causou um aumento (dependente da dose) da atividade metabólica de monócitos THP-1, provavelmente devido ao aumento da proliferação celular. Estes resultados preliminares terão ainda de ser confirmados num trabalho futuro, assim como o potencial imunomodulador destes extratos.

De um modo geral, o trabalho realizado nesta tese contribui para a descrição do potencial dos extratos de cogumelos na busca de compostos bioativos.

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| **53BP1** | P53 binding protein |
| **AECM** | Aqueous extracts from *C. militaris* |
| **Akt** | Protein Kinase B |
| **APAF-1** | Apoptotic protease activating factor 1 |
| **Bak** | Bcl-2 homologous antagonist/killer |
| **Bax** | Apoptosis regulator BAX also known as bcl-2-like protein 4 |
| **Bcl-2** | B-cell lymphoma 2 |
| **BrdU** | Bromodeoxyuridine (5-bromo-2'-deoxyuridine) |
| **BSA** | Bovine serum albumin |
| ***C. militaris*** | *Cordyceps militaris* |
| **CDKs** | Cyclin-dependent kinases |
| **DAPI** | 4',6-diamidino-2-phenylindole dihydrochloride |
| **DC** | Dendritic cells |
| **DISC** | Death-inducing signaling complex |
| **DNA** | Deoxyribonucleic acid |
| **DR** | Death receptor |
| **DSBs** | Double stranded breaks |
| **ECL** | Enhanced chemiluminescence |
| **ERK** | Extracellular signal-regulated Kinases |
| **FACS** | Fluorescence-activated cell sorting |
| **FasR** | Fas receptor |
| **FBS** | Fetal bovine serum |
| **Fips** | Fungal immunomodulatory proteins |
| **FITC** | Fluorescein isothiocyanate |
| **$GI_{50}$** | Concentration of extract that reduced cell growth to 50% |
| **HMW** | High-molecular-weight compounds |
| **HRP** | Horseradish Peroxidase |
| **IARC** | International Agency for Research on Cancer |
| **Ig** | Immunoglobulin |
| **JNK** | Jun N-terminal kinase |
| **LMW** | Low-molecular-weight compounds |
| ***M. esculenta*** | *Morchella esculenta* |

| | |
|---|---|
| **MA-1** | Synthetic analog of militarin |
| **MAPK** | Mitogen-activated protein kinase |
| **MMP-9** | Matrix metalloproteinase 9 |
| **mRNA** | Messenger ribonucleic acid |
| **mTOR** | Mammalian target of rapamycin |
| **NK** | Natural killer cells |
| **NOXA** | Phorbol-12-myristate-13-acetate-induced protein 1 |
| **NSCLC** | Non-small cell lung cancer |
| **PAP** | Polyadenylate polymerase |
| **PARP** | Poly ADP ribose polymerase |
| **PBS** | Phosphate buffered saline |
| **PFA** | Paraformaldehyde |
| **PI** | Propidium iodide |
| **PI3K** | Phosphatidylinositol 3-kinase |
| **PLS** | Polysacharidic |
| **PMA** | Phorbol 12-myristate 13-acetate |
| **RNA** | Ribonucleic acid |
| **RNase A** | Ribonuclease A |
| **ROS** | Reactive oxygen specie |
| **RT** | Room temperature |
| ***S. luteus*** | *Suillus luteus* |
| **SDS-PAGE** | Sodium dodecyl sulfate polyacrylamide gel electrophorese |
| **SE** | Standard error |
| **SRB** | Sulforhodamine B |
| **SSBs** | Single stranded breaks |
| **TBS** | Tris-Buffered Saline |
| **TCA** | Trichloroacetic acid |
| **TEMED** | Tetramethylethylenediamine |
| **TNFR1** | Tumor necrosis factor receptor 1 |
| **VEGF** | Vascular endothelial growth factor |
| **wt** | Wild-type |

# I

# GENERAL INTRODUCTION

## 1. Mushrooms

In 1992, Shu-Ting Chang and Philip G. Miles defined mushroom as "a macrofungus with a distinctive fruiting body, which can be found either below ground (hypogeous) or above ground (epigeous), large enough to be seen with the naked eye and to be picked by hand" (Guillamon *et al.* 2010). Today, the word "mushroom" usually refers to the fruiting body of the complete fungus which is produced by a mature mycelium (a vegetative part of fungus, that allows the feeding and growth of the mushroom (Cheung 2008)). The mushroom structure is represented in Figure 1.

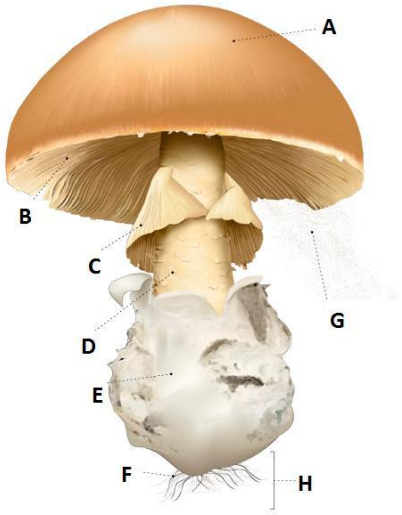


***Figure 1* –Mushroom structure.**
A) Cap, protect the gills; B) Gill, spore-producing part; C) Ring, remnant of a membrane that covers the gills of the immature mushroom; D) Stem, supports the mushroom cap; E) Volva, remnant of a membrane that covers the immature mushroom; F) Hypha, draws water and organic matter for the mushroom development; G) Spores, microscopic reproductive bodies; H) Mycelium, tangle of hyphae. The fruiting body is composed of A, B, C, D, E and G. [Adapted from http://www.ikonet.com/en/visualdictionary/static/us/edible_mushrooms in 21 July 2014]

Mushrooms belong to the kingdom *Myceteae,* being the majority included in the class "Basidiomycetes" and the others in the class "Ascomycetes" (Cheung 2008 *et al.* 2013). There are several mushrooms, presenting a variety of shapes and colors (Cheung 2008). Although it is not known how many mushroom species exist exactly on earth, its number has been estimated to be around 140.000, from which only a few (approximately 10%) have already been described. Furthermore, from the mushroom

species that have been already described, only few of them have been thoroughly studied (Hawksworth 2001, Wasser 2002, Khatua *et al.* 2013, Soares *et al.* 2013).

Although mushrooms are distributed worldwide, their production may be seasonal (Miles and Chang 1997). This may be due mainly to the fact that their fruiting bodies require the availability of more nutrients than the ones necessary for the production of asexual spores by microfungi (Cheung 2008).

Mushrooms may be divided in the four following categories, being included in one or more of them: i) edible, which includes all mushrooms which may be ingested without harm ( Table 1); ii) medicinal, which are considered to have medicinal applications; iii) poisonous, which are suspected or proven to be poisonous and iv) "other mushrooms", which includes a large amount of mushrooms whose properties remain unknown (Cheung 2008).

***Table 1* - Some edible mushrooms**

[Adapted from (Chang 2001)]

| **Genera of prime edible mushrooms** | |
|---|---|
| ***Ascomycetes*** | |
| *Morchella* | *Tuber* |
| ***Basidiomycetes*** | |
| *Agaricus* | *Agrocybe* |
| *Amanita* | *Armillaria* |
| *Auricularia* | *Boletus* |
| *Cantharellus* | *Calvatia* |
| *Clitocybe* | *Coprinus* |
| *Cortinarius* | *Dictyophara* |
| *Flammulina* | *Gloestereum* |
| *Grifola* | *Hericium* |
| *Hypholoma* | *Hypsizgus* |
| *Lactarius* | *Lentinula* |
| *Lepista* | *Lyophyllum* |
| *Marasmius* | *Pleurotus* |
| *Pholiota* | *Polyporus* |
| *Russula* | *Stropharia* |
| *Termitomyces* | *Tremella* |
| *Tricholoma* | *Volvariella* |

| **Species of commercially cultivated edible mushrooms** | |
|---|---|
| Species which have reached an industrial scale | |
| *Agaricus bisporus* | *Agaricus bitorquis* |
| *Flammulina velutipes* | *Lentinula edodes* |
| *Pleurotus ostreatus* | *Volvariella volvacea* |
| Others | |
| *Agaricus blazei* | *Auricularia auricular* |
| *Auricularia fuscosuccinea* | *Auricularia polytricha* |
| *Coprinus comatus* | *Dictyophora duplicate* |
| *Dictyophora indusiata* | *Grifola frondosa* |
| *Hericium erinaceus* | *Hypsizygus marmoreus* |
| *Lyophyllum ulmarium* | *Pholiota nameko* |
| *Pleurotus citrinopileatus* | *Pleurotus cornucopiae* |
| *Pleurotus cystidiosus* | *Pleurotus djamor* |
| *Pleurotus eryngii* | *Pleurotus florida* |
| *Pleurotus sajor-caju* | *Stropharia rugoso-annulata* |
| *Tremella aurantia* | *Tremella fuciformis* |
| *Volvariella diplasia* | *Volvariella esculenta* |

This thesis focuses mainly on the study of edible mushrooms with medicinal properties, namely antitumor and immunomodulatory.

## 1.1. Edible mushrooms and their nutritional properties

Edible mushrooms have been cultivated, consumed (both in fresh and processed forms, cooked or raw) and appreciated by many people around the world for thousands of years. In fact, mushrooms existed on earth even before man (as indicated by a fossil from the lower cretaceous period), raising the possibility that man have been using mushrooms as food since the most primitive times (Wani *et al.* 2010).

From all the species of mushrooms described worldwide, approximately 2000 have been described as edible, although (Lakhanpal and Rana 2005, Wani *et al.* 2010) only around 100 are experimentally grown, 50 are economically cultivated, approximately 30 are commercially cultivated and only fewer than 25 species are accepted as food (Chang 2001). In most countries, probably due to their unique flavour and texture, the consumption of cultivated mushrooms is well-established (Lindequist *et al.* 2005, Wong and Chye 2009, Kalyoncu *et al.* 2010, Wani *et al.* 2010, Ren *et al.* 2012, Tibuhwa 2012). Nevertheless, a specific group of edible mushroom species called "ectomycorrhizal" (that create symbiotic relations with their host plants) (Yun and Hall 2004) are only consumed seasonally, by local people (from the areas where those mushrooms grow), enthusiasts and gourmets (Chang 2001, Kalmiş *et al.* 2011). From all the species of mushrooms considered as food, the most popular, and usually mostly consumed around the world, are the *Agaricus bisporus* (also known as "button mushroom") and *Lentinus edodes*, (also known as "shiitake") (Mattila *et al.* 2000).

The majority of mushrooms are similar in terms of basic constitution: 90% water and 10% dry matter (Wani *et al.* 2010). From the nutritional point of view, mushrooms comprise a valuable source of healthy food, which is low in calories/energy (having only approximately 150 kJ per 100 g of fresh mushroom) (Cheung 2008, Kalač 2009, Khatua *et al.* 2013). Their nutritional value is due to their significant content in: antioxidant compounds, fibers, proteins (and enzymes), essential amino acids (containing all the nine essential amino acids for humans), carbohydrates (such as chitin, glycogen, mannitol and trehalose), lipids, minerals, vitamins and water, which can be efficient health promoters (Mattila *et al.* 2001, Lakhanpal and Rana 2005, Cheung 2008, Kalač 2009, Guillamon *et al.* 2010, Kalyoncu *et al.* 2010, Rathee *et al.*

2012, Tibuhwa 2012, Soares *et al.* 2013). In addition, it is also important to refer that they have a high content of digestible carbohydrates (approximately 50 to 60%) and that their protein content (between 27 to 48%) is equivalent to muscle protein (Khatun *et al.* 2012) and is higher when compared to most vegetables (Chang 2001, Lakhanpal and Rana 2005, Kim *et al.* 2008, Wani *et al.* 2010).

## 1.2. Pharmacological properties of edible mushrooms

Mushrooms are not only a source of nutritional properties but some of them also possess medicinal value (Sharma and Atri 2014).

Medicine and natural products have been, for a long time, intimately correlated through the use of traditional medicines and natural poisons (Ferreira *et al.* 2010). Food is no longer seen, and ingested, only as a source of required nutrients and to satisfy hunger, but also to prevent diseases and improve physical and mental well-being (Menrad 2003).

Interestingly, the great majority of the commercialized drugs has been produced from secondary metabolites of natural products or their chemically synthesized analogs (Li and Vederas 2009). Examples of such drugs which are used in the clinic are: i) penicillin, an antibiotic derived from *Penicillium*; ii) cyclosporine, an immunosuppressant derived from *Tolypocladium inflatum*, and iii) taxol, camptothecin and doxorubicin, anticancer drugs derived from *Taxus brevifolia*, *Cuscuta reflexa* and *Streptomyces peucetius,* respectively (Li and Vederas 2009, Kumar *et al.* 2013).

Mushrooms fulfill the criteria suggested by Baker in 1995, to consider natural species as sources of compounds with potential medicinal properties (Baker *et al.* 1995). These criteria are: a) evidence regarding the traditional use of the substance by indigenous populations; b) abundance of the species in nature; and c) sustainable use of the species (Ferreira *et al.* 2010).

Therefore, increased interest is being given to mushrooms (and their components), as a major untapped source of biologically active compounds with pharmacological

applicability which may be used in the development of potent new pharmaceutical products (Lakhanpal and Rana 2005, Rai *et al.* 2005, Cheung 2008, Ferreira *et al.* 2010, Kalmiş *et al.* 2011, Palacios *et al.* 2011, Khatun *et al.* 2012). Since mushrooms grow in darkness and dampness, in extremely competitive environments, they have the need to defend themselves from other microbes. This stimulates the accumulation of a diversity of secondary metabolites (such as phenolic compounds, terpenes and steroids) and of essential cell wall components (such as polysaccharides, β-glucans and proteins), several of them having important biological activities (Ferreira *et al.* 2010, Soares *et al.* 2013). Therefore, it is not surprising that mushrooms are capable of producing different important biologically active compounds which may be used in therapy, since they frequently need these natural protective substances for their survival (Ferreira *et al.* 2010, Soares *et al.* 2013).

Although the interest in the pharmacological properties of mushrooms appears to be recent, mushrooms have been used for generations in Oriental medicine. (Lindequist *et al.* 2005). Ancient civilizations like Greeks, Egyptians, Romans and Mexicans valued the therapeutic nature of mushrooms. Also Vedas, the oldest religious scriptures of Hinduism, mentioned the medicinal value of mushrooms, whereas the Romans considered mushrooms the “Food of the Gods” and the Chinese defined mushrooms as the “Elixir of life” (Mattila *et al.* 2000, Chang 2001). Their application in the Western hemisphere of the globe has been growing slightly over the last decades (Lindequist *et al.* 2005, Zong *et al.* 2012, Khatua *et al.* 2013). Mushroom preparations are considered a tonic with beneficial health effects (Lakhanpal and Rana 2005, Rai *et al.* 2005, Ferreira *et al.* 2010) and their extracts have already been proved to be generally well-tolerated with few, or any, side-effects (Roupas *et al.* 2012). In fact, many studies have suggested that the daily use of specific mushrooms (and/or their derivatives) in the diet may provide health benefits for the major functions of the human body, particularly since they presumably protect against oxidative damage, among other properties. They also have been described as prophylactic and healing promoters (Poucheret *et al.* 2006, Cheung 2010, Tibuhwa 2012).

Since mushroom are not (yet) directly used as a pharmaceutical product, their use as complementary medicine/dietary supplements may give them the connotation of

"nutraceuticals" (a substance which may be a food (or part of it) and is also beneficial for health, both in the prevention and treatment of diseases (Chang 1996, Lakhanpal and Rana 2005, Cheung 2008, Khatun *et al.* 2012, Soares *et al.* 2013).

From all the known species of mushrooms, approximately 1800 have been identified as having potential medicinal properties, and around 300 have recognized medicinal properties (Cheung 2008, Sharma and Atri 2014). For example, metabolites from Basidiomycetes were established as having pharmacological activity in several diseases such as: oxidation associated diseases, chronic inflammation, infections, diabetes, immune system disorders and cancer (Lakhanpal and Rana 2005, Poucheret *et al.* 2006, Cheung 2008). *Agaricus blazei, Cordyceps militaris, Ganoderma lucidum, Grifola frondosa, Hericium erinaceous, Lentinus edodes and Pleurotus ostreatus* (Table 1) are just a few of the cultivated Basidiomycetes whose medicinal value has been studied (Wasser 2002, Lakhanpal and Rana 2005, Zaidman *et al.* 2005, Khatun *et al.* 2012). However, the nutraceutical and medicinal value of a large number of species remains unknown (Lakhanpal and Rana 2005). Thus, the study of those less studied mushrooms (and of their compounds) is of great interest due to the variety of biomolecules with medicinal properties (Poucheret *et al.* 2006) they may possess.

Each mushroom has a particular composition (Palacios *et al.* 2011) that may have several applications. Some of the compounds which are found in mushrooms with biological activity include: polysaccharides, proteins, fats, ash, glycosides, alkaloids, volatile oils, tocopherols, phenolics, flavonoids, carotenoids, folates, ascorbic acid, enzymes, organic acids, dietary fibers, oligosaccharides, triterpenoids and mineral compounds. Polysaccharides are the best known and also considered the most potent mushroom-derived substances, being described as having antitumor and immunomodulating properties, among others (Mattila *et al.* 2000, Wasser 2002, Cheung *et al.* 2003, Borchers *et al.* 2004, Lakhanpal and Rana 2005, Zaidman *et al.* 2005, Patel and Goyal 2012).

Mushrooms have been described as having several properties such as: antioxidants (Tibuhwa 2012), antitumor (Lakhanpal and Rana 2005), antibacterial (Voravuthikunchai *et al.* 2010), antifungal (Wong *et al.* 2011), antiviral (Wang *et al.* 2007), antiparasitic

(Oluba *et al.* 2012), immunomodulatory (Sheu *et al.* 2007) anti-inflammatory (Qian *et al.* 2012), hepatoprotective (Soares *et al.* 2013). Mushroom extracts also have been proven as capable of reducing the blood pressure and cholesterol concentration, which are intimately related to some diseases such as hypercholesterolemia (Lakhanpal and Rana 2005). Mushrooms are also an excellent source of folic acid, preventing anemia (Khatun *et al.* 2012). Other effects such as cytostatic, immunosuppressive, antiatherogenic, hypoglycemic and antiallergic are also found in different mushrooms (Miles and Chang 1997, Lakhanpal and Rana 2005, Lindequist *et al.* 2005).

Although the therapeutic potential of several mushrooms has been already shown, their exact mechanisms of action, as well as the exact health benefits still require intensive investigation (Rathee *et al.* 2012).

Some specific pharmacological properties of mushrooms and of their isolated compounds will be further referred in the following subsections.

### 1.2.1. Mushrooms as natural source of compounds with antitumor activity

#### *1.2.1.1 Cancer: general remarks*

Cancer is one of the most serious health problems worldwide. It is the principal cause of death in economically developed countries and the second cause of death in developing countries, affecting individuals from different sexes, ages and races (Jemal *et al.* 2011). In 2012, the International Agency for Research on Cancer (IARC) through GLOBOCAN 2012, estimated a number of 14.1 million of new cases and of 8.2 million deaths related to cancer (Ferlay *et al.* 2013). It is expected that these numbers will continue to increase, not only due to the aging and growth of the world population, but also because of the risk behaviors of our society, which may lead to the development of cancer (Jemal *et al.* 2011, Siegel *et al.* 2012).

Data published in 2012 indicated that lung cancer was the most commonly diagnosed cancer worldwide (1.8 million cases, corresponding to 13.0% of the total number of cases) being also the most abundant cause of cancer-related death (1.6 million, 19.4%

of the total number of cases). In Europe, lung cancer was the second most commonly diagnosed type of cancer (15.9%; behind prostate cancer, which corresponded to 22.8%) and was also the major cause of cancer related deaths in 2012 (26.1%) (Ferlay *et al.* 2013).

Cancer is usually characterized by an abnormal and uncontrolled cellular growth (Kumar *et al.* 2012) which may be induced by several factors, all interacting with each other or in sequence. These factors may be: i) genetic alterations, which may be inherited or result from accumulated genetic mutations (leading to alterations in oncogenes, tumor-suppressor genes or microRNAs) ii) lifestyle or iii) environmental factors (Pharoah *et al.* 2004, Croce 2008). The abnormal and uncontrolled cellular growth may be reflected in changes in cell proliferation and/or cell death. In some cases, tumor cells acquire the ability to invade the surrounding tissue, to metastasize and invade different normal tissues and organs. In fact, these metastases are already described to be responsible for 90% of cancer-related deaths (Ruddon 2007, Zhong and Xiao 2009, Zong *et al.* 2012).

In 2000, a total of six hallmarks of cancer have been defined by Hanahan and Weinberg as being biological characteristics, shared by all cancers, which are acquired during the initiation and progression of tumors (Hanahan and Weinberg 2000). These included: i) sustained proliferative signaling, ii) resistance to cell death, iii) stimulation of angiogenesis, iv) unlimited replication, v) activation of invasion and metastasis and vi) evasion of growth suppressor signals. In 2011, this subject was revised by the same authors and the suggested number of cancer hallmarks increased to ten through the inclusion of: i) deregulation of cellular energetic, ii) genome instability and mutation, iii) tumor-promotion inflammation and finally iv) avoiding the immune destruction (Hanahan and Weinberg 2011) (Figure 2).


***Figure 2*- Hallmarks of cancer**
Representation of the acquired capabilities of tumor cells essential for tumor growth and progression [Adapted from (Hanahan and Weinberg 2011)]

#### 1.2.1.1.2. Cancer therapy

The most common strategies to treat cancer are based on surgery, chemotherapy, radiotherapy and immunotherapy (Fialho and Chakrabarty 2010). Nevertheless, all of them still present disadvantages and/or side effects and, for the majority of the cases, cancer treatment relies in the use a more than one of these approaches.

Surgery is considered the first-line therapy for solid tumors, which are accessible via surgical incision. However, due to the high rate of local recurrence in non-metastatic patients, there is usually the need to use adjuvant therapy, with chemotherapy being currently the most common (Tan *et al.* 2009).

Chemotherapy was introduced into the clinic in the 1970s and mainly targets the growth ability of cancer cells and induces cell death (Johnstone *et al.* 2002).

Indeed, as previously referred, the ability of cancer cells to resist and modify the mechanisms that influence cell growth and cell death are included in the hallmarks of cancer (Hunter *et al.* 2007).

Cell cycle consists of a sequence of events required for growth and cell division. As represented in Figure 3, it includes the interphase (the intermission between two mitosis phases), the mitosis (the process of nuclear division) and culminates in cytokinesis (cytoplasm division). The interphase consists in three different stages: i) G1 phase, the first period of growth during which the cell prepares for DNA synthesis), ii) S phase, in which DNA replication occurs and iii) G2 phase, during which the cell prepares for mitosis. DNA packaging and chromosome segregation occurs in mitosis and this stage includes prophase, metaphase, anaphase and telophase. Cells in G0 or quiescent cells are cells that are not growing or proliferating (Murray and Hunt 1993, Vermeulen *et al.* 2003, Berridge 2012).

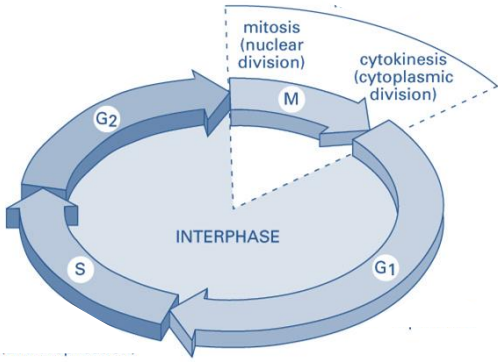


***Figure 3* – Cell cycle phases.**
G1- G1 phase; S- S phase (DNA replication); G2- G2 phase; M- M phase (divided in mitosis, a nuclear division, and cytokinesis, a cytoplasmic division). [Adapted from (Alberts *et al.* 2002)]

Usually, cell cycle is regulated between phases, at different checkpoints (evolutionarily conserved surveillance mechanisms) which allow to control and avoid abnormal events (Hartwell and Weinert 1989, Rowinsky 2005). In addition, cellular division in normal cells is limited in terms of number of successive cell cycles (Blasco 2007, Hanahan and Weinberg 2011). However, cancer cells seem to become "masters of their own destinies", being able to alter cell cycle and sustaining chronic proliferation (Hanahan and Weinberg 2000, Vermeulen *et al.* 2003, Blasco 2005, Hanahan and Weinberg 2011).

Cancer cells also usually resist to apoptotic cell death. This type of programmed cell death, contributes towards the maintenance of the homeostasis in living organisms (Wang *et al.* 2012). The cell has the ability to continuously check its integrity and once

this integrity is affected, a cascade of events is activated resulting in the cell self destruction (Darzynkiewicz *et al.* 1997, Khosravi-Far and Degli Esposti 2004) (Figure 4).

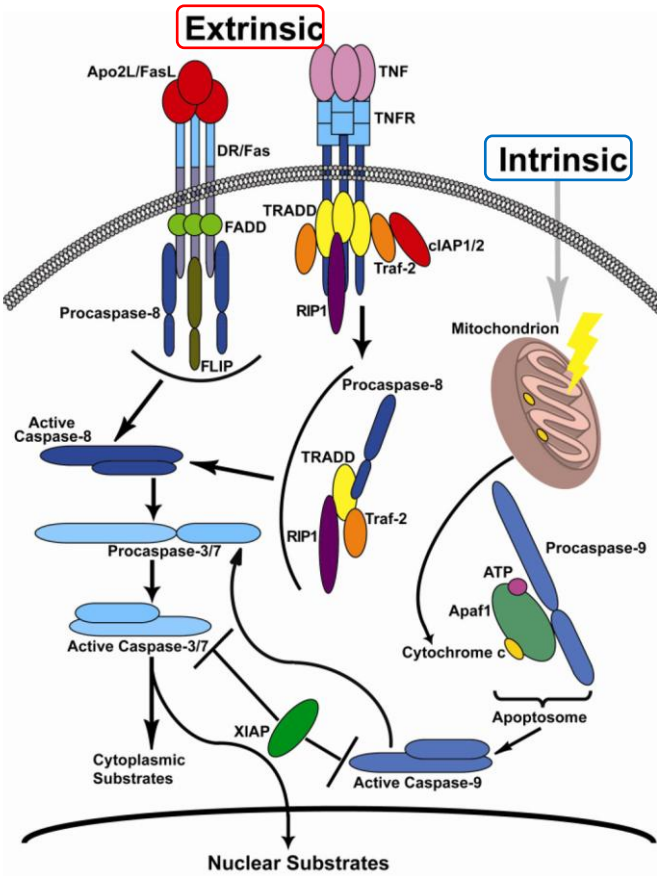


***Figure 4* - Apoptotic pathways**

[Adapted from (Wang *et al.* 2012)]

Induction of apoptosis may occur via two major pathways: the "intrinsic" (or mitochondrial pathway) and the "extrinsic" (or death-receptor pathway) (Hanahan and Weinberg 2011, Wang *et al.* 2012). In the intrinsic pathway, initiation occurs through the permeabilization of the outer mitochondrial membrane. Bax or Bak (Bcl-2 family proteins) dimerization regulates the formation of pores in the outer mitochondrial membrane, leading to the release of pro-apoptotic factors into the cytoplasm (such as cytochrome *c*), which associates with APAF-1 and procaspase-9, forming the caspase-9 activating complex. Active Caspase-9 then cleaves and activates caspases - 3, -6 and -7, leading to the cleavage of other substrates resulting in cell death (Jin and El-Deiry 2005, Wang *et al.* 2012). In the extrinsic pathway, the activation of receptors [Fas receptor (FasR), tumor necrosis factor receptor 1 (TNFR1), death receptor 4/death receptor 5 (DR4/DR5) and death receptor 3 (DR3)] occurs through the binding of their specific death ligands. This recruits the death-inducing signaling complex (DISC) to the cytoplasmic domain of the death receptors. DISC complex contains an adapter protein, which recruits procaspase-8 into the complex and, consequently, activates caspase-8 which then cleaves and activates caspase-3, leading to apoptosis (Jin and El-Deiry 2005, Wang *et al.* 2012).

Therefore, both cell cycle and apoptotic pathways are potential therapeutic targets for cancer treatment. Indeed, some of the conventional chemotherapeutic drugs are

known to reduce cancer cell growth by inducing cell cycle arrest and/or by inducing apoptosis (Hunter *et al.* 2007, Gerlinger and Swanton 2010).

Although conventional chemotherapeutic drugs are still the most frequent strategy in anticancer therapy, they exhibit many undesirable/adverse side-effects. In particular, since the majority of the chemotherapeutic drugs are not able to distinguish between normal and cancer cells, this result in the damage of normal growing cells. In addition, many chemotherapeutic agents activate apoptosis by inducing DNA damage, which may result in an increase the cellular mutation rate. This may, lead to the cancer drug resistance (Travis 2006, Hunter *et al.* 2007, Xu *et al.* 2013).

The development of anticancer drugs has been focusing in targets which are characteristic of tumor cells, and preferentially which are not expressed in normal cells (Brissette *et al.* 2006). These targeted therapies are therefore directed towards specific molecular targets, which may block one or more hallmark capabilities. The specificity of action of the more modern targeted therapies is an added value to cancer therapy, giving rise to comparatively less off-target effects and resulting in fewer nonspecific toxicity when compared to conventional chemotherapy (Hanahan and Weinberg 2011). However, several targeted therapies also result in relapses (Hanahan and Weinberg 2011). This may be explained by the fact that a targeted therapeutic agent can inhibit one signaling pathway, but is not able to completely shut off a hallmark capability. Furthermore, cancer cells might adapt by changing their dependence on a hallmark over another, and may also survive with residual function and adapt to the selective pressure imposed by the therapy applied, acquiring resistance and consequently causing a relapse in the cancer patient (Hanahan and Weinberg 2011).

Since tumors are a highly heterogeneous and highly dynamic mass of cells, they are extremely hard to destroy (Hunter *et al.* 2007, Gerlinger and Swanton 2010). Moreover, cancer is a multi-step process and may involve many different pathways (involving different epigenetic and genetic alterations). Therefore, different approaches have been studied in the last years to overcome cancer. In particular, the combination of therapies (targeting multiple pathways/targets) may offer higher

efficacy in treatment compared to therapy aiming at only one target (Csermely *et al.* 2005).

Based on the fact that the existing cancer therapies are still not fully effective, some of them having proved side effects and may have long-term consequences, the identification of novel and non-toxic therapeutic strategies is of prime concern (Kimura 2005, Eschenhagen *et al.* 2011, Kumar *et al.* 2013).

#### *1.2.1.2. Examples of mushrooms with antitumor activity*

Over the last decades, the potential use of mushrooms extracts in cancer therapy has emerged (Zaidman *et al.* 2005, Ferreira *et al.* 2010). Several studies, carried out in different human tumor cell lines, as well as in animal models, have shown that mushroom extracts may either be used individually (due to their antitumor properties), or as adjuvants, helping and supporting immune function in cancer patients, prolonging survival during treatment with conventional therapies (radio and chemotherapy) (Patel and Goyal 2012, Sharma and Atri 2014). About two hundred species of edible higher Basidiomycetes extracts have already been described as potential tumor cell growth inhibitors (Lindequist *et al.* 2005, Poucheret *et al.* 2006). Thus, the study of potential antitumor effects of mushrooms compounds and their mechanism of action are extremely important, since they may be a valuable source of potential antitumor drugs.

The first mushroom extract to be described as having antitumor properties was from *Boletus edulis*, in a study in which extracts of its fruiting bodies were tested against the Sarcoma 180 line in mice (Byerrum *et al.* 1957). Since then, several studies have described a vast number of compounds extracted from mushrooms with potential antitumor activity, acting through different mechanisms of action (Table 2). Compounds extracted from mushrooms which have potential antitumor activity may be divided in: i) low-molecular-weight compounds (LMW) such as quinones, cerebrosides, isoflavones, catechols, amines, triacylglycerols, sesquiterpenes, steroids, organic germanium and selenium and ii) high-molecular-weight compounds (HMW) such as homoglucans and heteroglucans, glycans, glycoproteins, glycopeptides,

proteoglycans, proteins and RNA-protein complexes (Ferreira *et al.* 2010). Polysaccharides can be extracted from different parts of mushrooms, like the mycelium and the fruiting body (Zhang *et al.* 2007). Glucans (HMW compounds) are considered the most important polysaccharides with antitumor potential (Ferreira *et al.* 2010, Ren *et al.* 2012). Nevertheless, the antitumor potential of mushroom polysaccharides does not seem to be a direct effect on the tumor but to be largely associated to their ability to modulate immune response (both innate and adaptive) (Borchers *et al.* 2004, Zaidman *et al.* 2005, Roupas *et al.* 2012).

***Table 2* - Some examples of extracts from mushrooms which have shown antitumor activity**
Adapted from (Popovic *et al.* 2013).

| Mushroom | Antitumor extracts | Cell line | Mechanism of action |
|---|---|---|---|
| *Amauroderma rude* | Hot water extract | MT-1<br>MDAMB231<br>4T1<br>MDAMB468<br>MCF7 | Induction of apoptosis |
| *Antrodia Camphorate* | Cold water extract | MDA-MB- 453<br>BT-474 | Inhibition of cell growth and induction of apoptosis through the induction of ROS, depletion of HER-2/neu, and disruption of the PI3K/Akt signaling pathway |
| | Fermentation culture | SKOV-3 | Modulation of HER-2/neu signaling pathway |
| *Antrodia cinnamonea* | Ethanolic extract | WEHI-3 | Inhibition of the proliferation and migration, MMP-9 protein expression reduction |
| *Clitocybe alexandri* | Ethanolic extract | NCI-H460 | S-phase cell cycle arrest |
| *Ganoderma lucidum* | Commercially available extract Reishi-Max GLpTM | mice injected with IBC cells | Decrease in mRNA and protein expression of molecules involved in PI3K/Akt/mTOR and MAPK signaling pathways |
| *Hericium erinaceus* | 50% ethanol extract | CT-26 mouse colon carcinoma cell | Suppression of ERK and JNK activation, inhibition of lung metastasis in vivo |

| | | | |
|---|---|---|---|
| *Lentinula edodes* | Aqueous extract | Hep-2 and HeLa | Induction of apoptosis |
| | Ethanol extract | HepG2 | Induction of apoptosis |
| *Pleurotus pulmonarius* | Hot water extract | Huh7, Hep 3B, SMMC-7721 and HepG2 | Inhibition of VEGF mediated autocrine regulation of PI3K/AKT |
| *Pleurotus sajor-caju* | Aqueous extract | Hep-2 and HeLa | Induction of apoptosis |
| *Suillus collinitus* | Methanolic extract | MCF-7 | Increase in p53 expression and induction of apoptosis |
| *Suillus luteus* | Methanolic extract | MCF-7, NCI-H460, AGS, HCT-15 | Increase in p53 expression and induction of apoptosis |
| *Tricholoma giganteum* | 80% ethanol extract | Ehrlich ascites carcinoma | Induction of apoptosis |

In some countries, such as China, Japan, Korea and other East Asian countries, some compounds extracted from mushrooms (namely Lentinan, Schizophyllan, Grifolan and Krestin, from *Lentinus edodes, Schizophyllum commune, Grifola fondosa* and *Trametes versicolor*) have already been approved for their use in clinical treatment in cancer patients (Lindequist *et al.* 2005, Lull *et al.* 2005, Poucheret *et al.* 2006, Ferreira *et al.* 2010).

Interestingly, it has been suggested that whole-mushroom extracts contain various compounds that may control tumorigenesis and carcinogenesis at different stages and/or may act at the same stage but through distinct mechanisms. The multiple medical properties of mushrooms might control tumorigenesis and carcinogenesis, since some of their compounds are actually capable of reducing tumor initiation by different mechanisms, such as enhancing the antioxidant activity (Lee and Nishikawa 2003). Also the immunomodulation ability documented in other compounds, predominantly in polysaccharides, may have an effect on the promotion and progression stages of carcinogenesis. Nevertheless, other mushroom compounds could inhibit/reduce promotion or progression by exerting direct cytotoxicity against tumor cells (Takaku *et al.* 2001). Therefore, whole-mushroom extracts could offer additional effects in the prevention and treatment of cancer (Lull *et al.* 2005) when applied as adjuvants to classical treatment against cancer, synergizing or potentiating conventional treatments as chemotherapy (Kimura 2005).

1.2.1.2.1. *Cordyceps militaris*

*Cordyceps militaris* (Figure 5) is a type of edible Ascomycete, also called "vegetable wasp and plant worm", reported as having potent medical properties (Park *et al.* 2005, Li *et al.* 2010). It is traditionally used in the Orient for longevity, endurance and vitality (Park *et al.* 2005) being also used as a crude drug and a folk tonic food for cancer patients (Das *et al.* 2010, Li *et al.* 2010, Rathee *et al.* 2012).

***Figure 5 - Cordyceps militaris.***
Adapted from (Zheng *et al.* 2011) and (Das *et al.* 2010), respectively.

This mushroom is an entomopathogenic fungus, a fungus which infects the larva, pupa or imago of insects (killing them), and its fruiting body grows on the larval, pupal or adult integument of the insect host (Park *et al.* 2005, Das *et al.* 2010). Although *C. militaris* has a worldwide distribution (probably due to the fact that it adapts to a wide range of host insects), its development requires strict growth environment conditions. Thus, the population density of *C. militaris* is very scarce in nature (Shih *et al.* 2007, Das *et al.* 2010, Shrestha *et al.* 2012). Although it is mainly an insect pathogenic fungus, it may also grow and finish its life cycle *in vitro* (in non-insect media) (Shrestha *et al.* 2012). Thus, artificial methods of cultivation and production of *C. militaris* fruiting bodies have been developed in the recent years. These cultivated *C. militaris*, besides allowing for a good model for the study of *Cordyceps* species (Shrestha *et al.* 2012), also possess pharmacological efficacy which is comparable to the natural ones (Rao *et al.* 2010). Moreover, the fact that the genome of *C. militaris* has already been sequenced (Zheng *et al.* 2011) allowed for a methodical study of the biology and

pharmaceutical properties of this mushroom and the identification of potential safety hazards (Zheng *et al.* 2011, Zheng *et al.* 2013).

The list of pharmacological activities described for *C. militaris* is vast and includes the following: anti-inflammatory (Yu *et al.* 2004, Won and Park 2005, Jo *et al.* 2010, Rao *et al.* 2010, Han *et al.* 2011, Ying *et al.* 2014), antioxidant (Yu *et al.* 2007, Jiang *et al.* 2011, Wu *et al.* 2011, Chen *et al.* 2013, Jeong *et al.* 2013), antitumor (Reis *et al.* 2013), anti-angiogenic (Won and Park 2005), antiproliferative (Liu *et al.* 1997) and antimetastatic (Shih *et al.* 2007), immunomodulatory (Zhou *et al.* 2002, Kim *et al.* 2006, Kim *et al.* 2006, Ohta *et al.* 2007, Kim *et al.* 2008, Lee and Hong 2011, Jeong *et al.* 2013, Xiong *et al.* 2013), antimicrobial, antibacterial (Ahn *et al.* 2000), antiviral (Muller *et al.* 1991, Ohta *et al.* 2007, Jiang *et al.* 2011), antifungal (Sugar and McCaffrey 1998, Shih *et al.* 2007), mitochondrial protective and anti-aging (Li *et al.* 2010), anti-malarial (Trigg *et al.* 1971), anti-fatigue (Mizuno 1999), protective of the activity of liver , kidney and lung (Yu *et al.* 2007) , among others (Liu *et al.* 1997, Das *et al.* 2010).

Regarding the antitumor activity of *C. militaris* and of some of its compounds, there are already several studies published in the literature, some of which will be referred in the following subsections.

#### *1.2.1.2.1.1. Water extracts from C. militaris*

Water extracts of *C. militaris* have shown ability to inhibit the growth of human tumor cells including: umbilical vein endothelial (HUVEC), human sarcoma (HT 1080) and melanoma (B16-F10) cells (Yoo *et al.* 2004). Furthermore, a water soluble fraction of *C. militaris* showed cytotoxic activity on gastric adenocarcinoma (SNU-1); colorectal adenocarcinoma (SUN-C4) and hepatocellular carcinoma (SNH-354) cells (Yue *et al.* 2013).

Treatment with an aqueous extract from this mushroom decreased leukemia cellular growth through induction of apoptotic cell death (Lim *et al.* 2004, Park *et al.* 2005). Likewise, induction of apoptosis following treatment with *C. militaris* water extracts

has also been shown in several other cell line models such as: human breast cancer (MDA-MB-231) cells (Jin *et al.* 2008), human promyelocytic leukemia (HL-60) (Lee *et al.* 2006) and human lung carcinoma (A549) cells (Park *et al.* 2009).

Furthermore, hot water extracts from this mushroom also exhibited an antiproliferative effect on tumor cells, and prolonged the overall survival in mice bearing ascitic tumors (Lee *et al.* 2003) or sarcoma-180 solid tumors (Lee and Lee 2004). Also, crude water-soluble polysaccharides extracted from the fruiting bodies of this species suppressed the growth of a murine melanoma B16-F10 cell line (Lee and Hong 2011).

Interestingly, treatment of xenografts in nude mice of NCI-H460 cells (non-small cell lung cancer cells, NSCLC) with an aqueous extract of *C. militaris* resulted in tumor shrinkage and increased mice lifespan (Park *et al.* 2009).

#### *1.2.1.2.1.2. Methanolic extract from C. militaris*

Regarding the methanolic extracts from *C. militaris* and their antitumor activity, the existing information is still limited. In fact, only two studies seem to have been published on this subject. Reis and collaborators showed that a methanolic extract of *C. militaris* fruiting bodies (the one used in this thesis) inhibited proliferation of MCF-7 (breast adenocarcinoma), NCI-H460 (NSCLC), HCT-15 (colon adenocarcinoma) and HeLa (cervical) cell lines (Reis *et al.* 2013), without affecting non-tumor liver primary cells (PLP2). Likewise, Liu and collaborators also observed that methanolic extracts of fermented mycelia and fruiting bodies of *C. militaris* inhibited the growth of HCT-116 (colon adenocarcinoma), A549 (lung adenocarcinoma), MGC-803 (gastric adenocarcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast adenocarcinoma) and HL-60 (promyelocytic leukemia) cell lines (Liu *et al.* 2014).

Nevertheless, the mechanism(s) of action of the methanolic extract of *C. militaris*, in particular of its fruiting body, remain unknown. These may be due to some of its potential components, namely cordycepin.

*1.2.1.2.1.3. Some antitumor compounds isolated from C. militaris*

Some of the compounds extracted from *C. militaris* with antitumor activity have already been identified and characterized.

Cordycepin (which is also isolated from other *Cordyceps*, Figure 6) is one of the compounds isolated from *C. militaris*. In fact, it is considered by many authors to be a key compound for the antitumor activity of *C. militaris*, although there are other studies that showed other isolated compounds with antitumor activity (Liu *et al.* 2014). Cordycepin inhibits the activity of polyadenylate polymerase (PAP) and may abrogate mRNA synthesis during the polyadenylation, resulting in cell death (Thomadaki *et al.* 2005, Wu *et al.* 2007, Jeong *et al.* 2011). It has been shown to be an inducer of apoptosis (Thomadaki *et al.* 2008, Chen *et al.* 2010, Jeong *et al.* 2011, Baik *et al.* 2012, Ko *et al.* 2013) and also to affect cell cycle (Thomadaki *et al.* 2005, Wu *et al.* 2007). In particular, it was found to cause induced G2/M cell arrest in OEC-M1 (oral cancer) (Wu *et al.* 2007) and in MCF-7 (breast cancer), and to induce S cell arrest in HeLa (cervical cancer) cells (Thomadaki *et al.* 2005). In addition, *in vivo* studies showed that Cordycepin decreased cell growth of B16-BL6 mouse melanoma (Yoshikawa *et al.* 2004) and growth of hematogenic metastasis of mouse melanoma cells and mouse lung carcinoma cells (Ko *et al.* 2013).

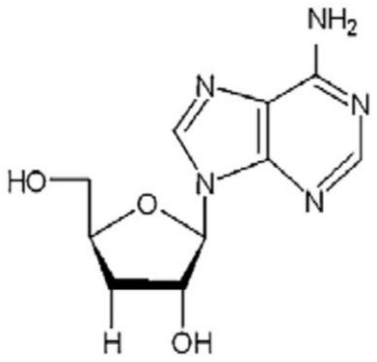


***Figure 6* - The chemical structure of cordycepin produced by *C. militaris*.**
[Extracted From (Tuli *et al.* 2013)]

The peptide Cordymin is another of the compounds isolated from *C. militaris* which has shown anti-proliferative activity in MCF-7 breast cancer cells (Wong *et al.* 2011). Also, MA-1, a synthetic analog of militarin, inhibited the viability of several cell lines (namely A549, H358, and NCI-H460) by inhibiting cell growth and inducing apoptosis (Yoon *et al.* 2013). In addition, CMP-1, a low-molecular-weight polysaccharide inhibited proliferation of HT-29, HeLa, HepG2 and K562 cells (Jing *et al.* 2014).

### 1.2.2. Mushrooms as natural source of compounds with immunomodulatory activity

The stimulation of immune response leads the improvement of body's defense mechanisms, therefore playing a crucial role in the prevention and/or treatment of diseases, including cancer (Zitvogel *et al.* 2008, Zhou *et al.* 2009, Lee and Hong 2011).

The strategy behind the use of immunomodulation in therapy consists of identifying aspects of the host response that can be changed, resulting in an increase of the desired immune response (Zaidman *et al.* 2005).

Immunomodulators, also known as "biological response modifiers", "immunopotentiators" or "immunostimulants", are able to stimulate or inhibit the immune system (Moradali *et al.* 2007, Rathee *et al.* 2012). This enhancement or suppression of immune responses is dependent on the dose, route of administration, time of administration, mechanism of action and site of activity of the immunomodulators (Moradali *et al.* 2007, Rathee *et al.* 2012).

#### *1.2.2.1 The immune system: general remarks*

The immune system is a complex system (involving different organs, tissues, cells and their soluble products) which plays the important and indispensable role of protection and defense, against non-self entities like infectious agents (such as bacteria, microbes, viruses, toxins and parasites) as well as maintenance of the body homeostasis in response to endogenous signals (Mak *et al.* 2005). This complex system

recognizes attacks and destroys everything that is considered as harmful to the body (Mak *et al.* 2005). When the immune system fails, several pathologies or adverse reactions may be triggered.

An abnormal immune system may attack the body's cells and tissues resulting in autoimmune diseases. It may also react against an antigen that is generally harmless, culminating in allergic reactions. When the immune reaction is too strong or dysregulated, it may result in hypersensibility against the antigen. Finally, when the immune system is decreased (naturally or induced by external factors) immunodeficiency occurs in the host, compromising the natural health balance (Mak *et al.* 2005).

The immune response is affected by several factors (such as age, gender, endocrine factors, stress, environment and health condition (Cohen *et al.* 1991, Brabin 2002, Srinivasan *et al.* 2005)) and is divided in two different types of response: the innate and the adaptive, which are not mutually exclusive. These two different types of immune response differ in degree of specificity and also in the mechanisms activated (Mak *et al.* 2005). The innate immunity response provides for an immediate response, although being less-specific than the adaptive response. On the other hand, the adaptive response is activated by the innate response, providing a stronger and highly specific response, being able to develop memory (recalling specific intruders and producing a faster and stronger response in later challenges (Litman *et al.* 2005)).

In innate immunity, the monocytes, macrophages, neutrophils, dendritic cells (DC) and natural killer (NK) cells respond quickly to molecular patterns and some of them (monocytes, macrophages and DC) present particular antigens. This response does not result in long-term immunity; however, it may lead to the activation of long-lasting pathogen-specific response of the adaptive immune system (Lin *et al.* 2009). Innate immune cells recognize and destroy atypical cells. These events occur by the stimulation of macrophages (with their activation) and NK cells in order to produce cytokines, interleukins and other inflammatory regulator molecules that are targeted towards destroying abnormal cells (Moradali *et al.* 2007, van Griensven and Verhoeven 2013).

Macrophages are mononuclear differentiated cells originated from blood monocytes, which are key players in immune response since they respond quickly against pathogens. When macrophages are activated, they produce inflammatory regulators, such as cytokines, COX-2, prostaglandins and nitric oxide, which in turn activate the MAPK dependent signaling pathways and NF-κB dependent transcription regulation (van Griensven and Verhoeven 2013). In innate immunity, when reaching the target tissues (Mosser and Edwards 2008), they may have different functions, such as killing, phagocyting and removing pathogenic microbes. Macrophages also play an important role in the adaptive immunity, by presenting antigens to lymphocytes and by regulating lymphocyte activation and proliferation (Elhelu 1983, Shin *et al.* 2010).

#### *1.2.2.2. Examples of mushrooms with immunomodulatory activity*

The therapeutic effects of several mushrooms may be related (directly or indirectly) to the enhancement of immunity of the host (Roupas *et al.* 2012). In the last decades, the study of mushrooms as natural sources of compounds with low toxicity and high capacity to activate the immune system has been increasing (Mizuno and Nishitani 2013). Moreover, their ability to modify the immune response has already been proven (Zaidman *et al.* 2005, Hilszczańska 2012). In fact, mushroom-isolated compounds with immunodulatory potential exert their activity through: i) mitogenicity, ii) activation of alternative complement pathway or iii) stimulation of immune effector cells from the innate and adaptive immunity [such as hematopoietic stem cells, macrophages, lymphocytes, T cells (T helper cells and T cytotoxic cells), dendritic cells, and natural killer cells resulting in the production of cytokines] (Lull *et al.* 2005, Moradali *et al.* 2007).

Some of the mushroom-extracted compounds considered as immunomodulators are: polysaccharides, glycoproteins, glycopeptides, proteoglycans, fungal immunomodulatory proteins (Fips) and triterpenoids. These molecules are able to affect the proliferation and differentiation of immune cells, and also of cytokines and interleukins, thus enhancing the innate and adaptive immune responses (Moradali *et al.* 2007). Polysaccharides act via the innate immunity, mediated by the release of cytokines which later will stimulate the adaptive immunity (Brown and Gordon 2003,

Volman *et al.* 2010), increasing the host ability to resist against virus, bacteria and other intruders (Zheng *et al.* 2005). β-Glucans are considered the most important polysaccharides with immunomodulary activity without presenting adverse effects (Zheng *et al.* 2005, Ramberg *et al.* 2010, Mizuno and Nishitani 2013). β-Glucans have been proved to increase phagocytosis activity, nitric oxide production, catalase activity of macrophages and to reduce tumor formation in a mouse model (Zheng *et al.* 2005, Ramberg *et al.* 2010).

Different studies have proven that different mushroom extracts and compounds exert effect on macrophages, by activating them to produce mediators (Lull *et al.* 2005).

***Table 3* - Some immunomodulatory compounds extracted from mushrooms**
[Adapted from (Lindequist *et al.* 2005)]

| Mushroom | Immunomodulator compound |
|---|---|
| ***Agaricus brasiliensis*** | FIo-a-β<br>FA-2-b-Md |
| ***Cryptoporus volvatus*** | H-3-B |
| ***Flammulina velutipes*** | Flammulin |
| ***Ganoderma lucidum*** | GLP(AI), Ganopoly, Ganoderans<br>Protein LZ 8 |
| ***Grifola frondosa*** | MD-fraction<br>Grifolan |
| ***Lentinus edodes*** | Lentinan, KS-2<br>LEM |
| ***Lentinus strigellus*** | Polysaccharides |
| ***Phellinus linteus*** | Polysaccharides |
| ***Schizophyllum commune*** | Schizophyllan, Sonifilan |
| ***Sparassis crispa*** | SCG |
| ***Trametes versicolor*** | PSK, PSP |
| ***Tremella fuciformis*** | Tremellastin |
| ***Tricholoma lobayense*** | Polysaccharide–peptide complex |
| ***Tricholoma mongolicum*** | Lectin |

FIo-a-β and FA-2-b-Md: water-soluble fractions from the fruiting body of *Agaricus blazei*; H-3-B: A water-soluble (1 → 6) -branched (1 → 3)-β-d-glucan, isolated from a hot-water extract of the fruiting bodies of *Cryptoporus volvatus*; GLP(AI): polysaccharide fraction of hot-water extract of the mycelium of *Ganoderma lucidum*; MD-fraction: Maitake (*Grifola frondosa*) D-Fraction; KS-2 : polysaccharide extracted from culture mycelia of *Lentinus edodes*; LEM: Complex mixture of polysaccharides and lignin from *Lentinus edodes* mycelia; SCG: purified β-glucan preparation from *Sparassis crispa;* PSK: semi-purified polysaccharide peptide Krestin from Trametes versicolor; PSP:polysaccharopeptide from *Trametes versicolor*

1.2.2.2.1. *Suillus luteus*

*Suillus luteus* (Figure 7) in an edible mycorrhizal mushroom (decomposers of organic matter, forms mycorrhizal associations with plant roots) species from Northeast Portugal. The symbiosis of this mushroom with the vegetation and soils may possibly influence the production of metabolites, including bioactive compounds (Crabtree *et al.* 2010, Reis *et al.* 2011). There are already some published studies showing the biological activity of *S. luteus* extracts, including antitumoral (Santos *et al.* 2013, Jia *et al.* 2014, Santos *et al.* 2014) and antioxidant (Reis *et al.* 2011). However, its potential as immunomodulator has not been previously addressed.

***Figure 7 - Suillus luteus***
Adapted from (Ostry *et al.* 2011).

1.2.2.2.2. *Morchella esculenta*

*Morchella esculenta* (Figure 8), one of the most widely appreciated (although rare) wild edible mushrooms, is characterized by a hollow fruit body. This mushroom has a huge potential value in China where it is used in traditional medicine for indigestion, excessive phlegm and shortness of breath (Duncan *et al.* 2002, Prasad *et al.* 2002, Cui *et al.* 2011, Fu *et al.* 2013, Li *et al.* 2013).

The main nutritional components of *M. esculenta* are protein, fat, fiber, ash and carbohydrates (Xu *et al.* 2008), with its principal biological active compounds being polysaccharides, proteins, trace elements, dietary fiber and vitamins (Cui *et al.* 2011).

***Figure 8- Morchella esculenta***
Adapted from (Ostry *et al.* 2011)

Previous studies have shown some of the biological activities of *M. esculenta*, namely antioxidant (Mau *et al.* 2004, Elmastas *et al.* 2006, Meng *et al.* 2010) anti-inflammatory (Nitha *et al.* 2007), antimicrobial (Kalyoncu *et al.* 2010, Heleno *et al.* 2013) and antitumor (Nitha *et al.* 2007).

Additionally, some work has been previously published on the immunomodulatory activity of some of the *M. esculenta* extracts or isolated compounds. Indeed, two water-soluble polysaccharides (MEPs) from the fermentation broth of *M. esculenta* were shown to significantly modulate the immune activity by selectively activating T cells and purified lymphocytes (Cui *et al.* 2011). In addition, galactomannan, a compound extracted from *M. esculenta*, enhanced macrophage activation (Duncan *et al.* 2002).

Overall, it is clear that several edible mushrooms possess some attractive compounds which have tonic and medicinal qualities. Although there are already several studies on the antitumor and immunomodulatory potential of mushrooms, there are still many mushrooms species whose properties have not yet been thoroughly studied, in particular their mechanism of action. This may be important for the identification of new therapeutic strategies.

II

# AIMS

As already mentioned, several mushrooms are an unlimited source of compounds with recognized antitumor and immunomodulatory activities. However, the potential of some mushrooms remains unknown, and for others their mechanism of action is still unidentified.

A recent study from external collaborators of the supervisors of this study has identified a methanolic extract from *Cordyceps militaris* as an inhibitor of the growth of human tumor cell lines, particularly of a non-small lung cancer cell line (NCI-H460) (Reis *et al.* 2013). Nevertheless, its mechanism of action remained unknown. Therefore, the first aim of the present work was to study the mechanism of action of the methanolic extract of *Cordyceps militaris* in the NCI-H460 cells, specifically regarding effect on:

- Cellular proliferation
- Cell cycle profile
- Cellular apoptosis
- DNA damage

This cell line was chosen based on the fact that: i) in the above mentioned study it was one of the most sensitive cell lines to the effect of the extract; ii) it is representative of a type of cancer that is still responsible for many deaths and iii) it has *wild-type* p53 which would allow studying p53-dependent mechanisms of action.

The second aim of this work was to study the immunomodulatory activity of two mushrooms extracts: i) a polysachararidic (PLS) extract of *Suillus luteus*, and ii) a phenolic extract of *Morchella esculenta*. For this, the THP-1 human monocytic cell line was used as a model, since these cells behave like monocytes and, upon stimulation they may differentiate into machrophages (immunologically active cells) (Auwerx 1991). In particular, it was intended to analyse the effect of the referred extracts in THP-1 cellular proliferation and differentiation .

III

# MATERIAL AND METHODS

## 3.1. Mushroom extracts

The following mushroom extracts were used: i) a methanolic extract of *Cordyceps militaris*, ii) a polysaccharidic (PLS) extract of *Suillus luteus* and a phenolic extract of *Morchella esculenta*. All the extracts used in this work were prepared and kindly provided by Prof. Doctor Isabel Ferreira from Instituto Politécnico de Bragança (IPB), Portugal.

The methanolic extract of *C. militaris* had been previously obtained by collaborators of the group from cultivated fruiting bodies of *C. militaris* (L.) Link (strain: MCI 10304, Meshtech Cordyceps Institute), as published in Reis *et al.* (2013). A stock solution at 8 mg/mL in $H_2O$ was stored at -20ºC.

The polysaccharidic (PLS) extract of *S. luteus* was obtained from samples of *S. luteus* (L.: Fries) Gray, collected in Bragança (Portugal) by the same collaborators of the group (Santos *et al.* 2013).

Like the other extracts, also the phenolic extract of *Morchella esculenta* had been obtained by the same collaborators. This extract was obtained from specimens of *Morchella esculenta* (L.) Pers. collected in Bragança (Portugal) (Heleno *et al.* 2013).

The stock solution of PLS extract from *Suillus luteus* and phenolic extract from *Morchella esculenta* were prepared at 50 mg/mL in $H_2O$ and stored at -20$^{o}$C.

## 3.2. Cell Culture

All the procedures involving cell culture were carried out in a biosafety level II laminar flow cabinet (Telstar Bio II Advance).

The following cell lines were used: NCI-H460 (NSCLC, adherent) and THP-1 (human leukaemia monocytic cell line from peripheral blood; in suspension). Cells were routinely maintained in culture in 25 $cm^2$ or 75 $cm^2$ tissue culture flasks, in RPMI

(Roswell Park Memorial Institute) 1640 medium with UltraGlutamine I and 25mM HEPES (Lonza), supplemented with 10% fetal bovine serum (FBS; Biowest), and incubated at 37°C in a humidified atmosphere with 5% $CO_2$. NCI-H460 cells were split twice per week (when confluency reached approximately 80%). For this, cell medium was discarded and cells washed with phosphate buffered saline (PBS; 1ml) to remove trypsin inhibitors. Trypsin-EDTA (0.5ml; Lonza) was then added and incubated for 10 min at 37 °C to allow cell detachment (Gilbert *et al.* 2006). Complete medium was added to inactivate trypsin and the following procedures were carried out: i) when starting an experiment, cells were centrifuged, medium discarded and cells resuspended in fresh medium at the desired concentration and ii) for routine maintenance, cells were diluted 1:20 and seeded into a new 25 $cm^2$ flask.

Regarding the THP-1 suspension cells, cells were sub-cultured twice per week. For this, cells were centrifuged at 1500 rpm from 5 minutes and resuspended in fresh medium at a 3 x $10^5$ cell/ml.

To induce THP-1 differentiation from monocytes into macrophages, cells were incubated with 20 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), for 18 h at 37 °C and 5% $CO_2$. After incubation, medium with PMA was removed, cells were washed with PBS, and further incubated for 24 h in fresh medium without PMA to induce maturation (Tsuchiya *et al.* 1982, Takashiba *et al.* 1999, Roussaki *et al.* 2012).

### 3.2.1. Cell number and viability - Trypan blue exclusion assay

Cell number and viability were evaluated using Trypan blue exclusion assay. This dye allows distinguishing viable cells (which exclude the dye, since their cell membrane is intact) from death cells (which take up the dye, since the membrane permeability is already compromised). Cell suspension was mixed 1:1 with a trypan blue solution (0.2%; Sigma-Aldrich) and loaded into a Neubauer chamber. The number of cells (stained and unstained) was counted under a bright-field microscope (Nikon Alphaphot 2 YS2) and cell density (number of cells/mL) was determined using the following formula: number of viable cells/field x dilution factor x $10^4$. In addition, the percentage

of viability (corresponding to the ratio of the number of viable cells and the number of total of cells x 100) was also determined.

All experiments were performed with exponentially growing cells which presented viabilities higher than 90%.

Throughout all the experiments, cells were daily observed under an inverted microscope (Nikon Eclipse TS100) to assess their morphological features and to avoid contaminations.

## 3.3. Analysis of the effect of methanolic extract of *Cordyceps militaris* in NCI-H460 cells

In this part of the study, the NCI-H460 cell line was used. Cells (1 x $10^5$/well) were plated in 6-well plates (TPP Techno Plastic Products) and incubated for 24 hours at 37 $^oC$ in 5% $CO_2$ to allow cell adhesion. After 24 h incubation, cells were treated with the methanolic extract either at its $GI_{50}$ (concentration of extract that reduced cell growth to 50%), previously determined to be approximately 50 µg/mL (Reis *et al.* 2013), with twice this concentration (100 µg/mL), or with half the $GI_{50}$ concentration (½ $GI_{50}$; 25 µg/mL). The following controls were also included: Blank (cells treated with medium only) and vehicle control ($H_2O$, in an equivalent concentration to concentrations of extract used). Following 48 h treatment, the medium was removed (but not discarded) and cells were trypsinised. The previously removed medium was then used to inactivate trypsin and the cell suspension was centrifuged at 1200 rpm for 5 minutes in a centrifuge (MPW 350R, Med. Instruments). Cell pellets were further processed according to the procedures described below.

### 3.3.1. Analysis of cellular proliferation with the BrdU incorporation assay

For the analysis of cellular proliferation, the BrdU (5-bromo-2'-deoxyuridine) incorporation assay was carried out as follows. Following 47 h incubation with the extract, cells were incubated for 1 h with 10 µM BrdU (Sigma Aldrich) at 37 °C and 5% $CO_2$. Cell pellets were then obtained (as previously described in section 3.3 "Analysis of the effect of methanolic extract of *C. militaris* in NCI-H460 cells") washed in PBS and fixed in 4% paraformaldehyde (PFA; Merck) in PBS for 30 minutes at room temperature. Cells were then centrifuged and cell pellets were resuspended in PBS and kept at $4^{o}$C until processed. For analysis, cell cytospins were prepared by centrifuging cells at 500 rpm for 5 min in a cytospin centrifuge (Shandon Cytospin 3). Cells were washed 3 times with PBS and incubated in 2 M HCl at room temperature for 20 minutes, for DNA denaturation. Subsequently, cells were washed twice in PBS, twice in PBS-T-B [PBS, 0.5% Tween 20 (Promega), 0.05% BSA (Sigma Aldrich)] and incubated with mouse anti-BrdU antibody (1/10 dilution in PBS-T-B; Dako) for 1 hour at room temperature. After being washed twice in PBS-T-B, cells were incubated with anti-mouse-Ig-FITC (diluted 1/100 in PBS-T-B: Dako) for 30 minutes, at room temperature (in the dark). Cells were then washed again twice with PBS-T-B and slides were prepared with Vectashield mounting medium with 4',6-diamidino-2-phenylindoledihydrochloride (DAPI) (Vector laboratories). BrdU incorporation was analyzed using a fluorescence microscope (Leica DM2000). A semi-quantitative evaluation of the levels of proliferation was carried out by counting at least 500 cells per treatment and determining the percentage of FITC-stained nuclei in the total DAPI-stained nuclei.

### 3.3.2. Analysis of cell cycle profile by Flow cytometry

Cell cycle profile was analyzed by flow cytometry after staining with propidium iodide (PI). Cell pellets (obtained as previously described in section 3.3 "Analysis of the effect

of methanolic extract of *C. militaris* in NCI-H460 cells") were immediately fixed in 2 mL ice-cold 70% ethanol (Carlo Erba Reagents) and stored at 4$^{o}$C for at least 12 h before being analyzed. Cells were then centrifuged for 5 min at 1200 rpm and resuspended in a solution of PBS with 5 µg/mL PI (Sigma-Aldrich) and 0.1 mg/mL RNase A (Sigma-Aldrich). Cellular DNA content was measured by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, USA). The percentage of cells in the G1, S and G2/M phases of the cell cycle (as well as the percentage of cells in the sub-G1 peak) were determined using the FlowJo 7.6.5 software (Tree Star, Inc., Ashland, OR, USA) after cell debris and aggregates exclusion and plotting at least 20.000 events per sample.

### 3.3.3. Analysis of apoptosis by Flow cytometry

For the analysis of apoptotic cell death, the Annexin V-FITC Apoptosis Detection kit (eBioscience Dx Diagnostics) was used. Cell pellets (obtained as previously described in section 3.3 "Analysis of the effect of methanolic extract of *C. militaris* in NCI-H460 cells") were resuspended in 400 µL binding buffer (previously diluted 1:4 in water). Samples were then divided in two parts, the first corresponding to the "autofluorescence" and the second corresponding to the incubated with Annexin V-FITC/PI which was further processed as follows. Cell suspension (195 µl) was incubated with Annexin V-FITC (5 µL) for 10 min at room temperature, in the dark. Propidium iodide (10µL) was then added and further incubated at room temperature for 2 min in the dark. Samples were immediately analyzed for the staining of Annexin V-FITC/PI by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences, USA) plotting at least 20.000 events per sample. Data was analyzed using FlowJo 7.6.5 software (Tree Star, Inc., Ashland, USA) after cell debris exclusion.

## 3.3.4. Analysis of protein expression

### 3.3.4.1. Total protein extraction and protein quantification

Cell pellets (obtained as previously described in section 3.3 "Analysis of the effect of methanolic extract of *C. militaris* in NCI-H460 cells") were lysed in Winman´s Buffer (50mM EDTA; 1.5M NaCl; 1% NP-40; 1 M Tris-HCl) supplemented with EDTA-free protease inhibitor cocktail (Roche), for 45 min at 4$^{o}$C with agitation. Cell lysates were centrifuged at 4$^{o}$C for 10 minutes at 13.000 rpm and the pellet discarded. Protein lysates were kept on ice until quantification (or stored at -20 $^{o}$C).

The quantification of the total protein content was carried out using "Bio-Rad´s DC Protein Assay" kit (Bio-Rad). For this, a standard curve was prepared, using serial dilutions of bovine serum albumin (ranging from 0 µg/mL to 3000 µg/ml). Briefly, a volume of 5 µl of protein lysate (or standards) was platted (in duplicate) in a 96 well plate (Orange Scientific). To each well, 25 µl of reagent A´ (prepared by adding 20 µl of reagent S to 1mL of reagent A) and then 200 µl of reagent B were added. The plate was gently agitated and incubated for 15 min at RT in the dark. Absorbance was then read at 655 nm in a multiplate reader (BioTek® Synergy HT).

### 3.3.4.2. Western blot

For the analysis of protein expression, Western blot was carried out. For this, protein samples were prepared by mixing protein lysates (30 µg) with loading buffer [10% sodium dodecyl sulfate (SDS), 1.0 M Tris-HCl, 50% Glycerol, 10% β-mercaptoethanol, 1% bromophenol blue]. After being denaturated at 95 oC for 5 minutes, proteins were loaded and electroforetically separated in a 12% Bis-Tris SDS-PAGE gel (Sambrook *et al.* 1982). A protein molecular weight marker (BioRad) was also loaded in each gel. Proteins were electrophoretically transferred to a Hybond-C Extra nitrocellulose membrane (Amersham Biosciences) at 100 V during 1 h and 30 minutes. Membranes were blocked for 1 hour at RT (or overnight at 4 $^{o}$C) in TBS-T [TBS (137 mM NaCl; 3 mM

KCl; 25 mM Tris-Base; $H_2O$) with 0.1% of Tween-20 (Promega)] containing 5% skimmed milk (Molico, Nestlé). After blocking, membranes were incubated with the following primary antibodies: rabbit anti-PARP (1:2000; Santa Cruz Biotechnology); mouse anti-p53 (1:200; Neomarkers); mouse anti-p21 (1:250; Calbiochem); rabbit anti-PH2Ax (1:200; Santa Cruz Biotechnology) or goat anti-actin (1:2000; Santa Cruz Biotechnology). Incubations were for 1 h 30 min at RT (for PARP, p53, P-H2Ax and actin) or overnight at 4$^{o}$C (for p21). Membranes were washed with TBS-T and then further incubated with the respective following secondary antibodies: goat anti-rabbit-IgG-HRP; goat anti-mouse-IgG-HRP or donkey anti-goat-IgG-HRP (1:2000; Santa Cruz Biotechnology) for 1 hour at RT. Protein signals were detected by chemiluminescence using the ECL Western blot Detection kit (GE Healthcare), the Amersham Hyperfilm ECL (GE Healthcare) and Kodak's GBX developer and fixer (Sigma-Aldrich). For the analysis of some proteins, membranes were striped for 30 minutes at 50 $^{o}$C in stripping buffer (1 M Tris-HCl, 14.3 M β-Mercaptoethanol and 10 % SDS), washed in TBS-T, blocked in 5 % skimmed milk (Molico, Nestlé) in TBS-T, as previously described and reprobed with the specific antibody. The intensity of the bands obtained in each film was quantified using the Quantity One software (BioRad).

### 3.3.5. Analysis of 53BP1 expression by Immunofluorescence

Cells pellets (obtained as described in section 3.3 "Analysis of the effect of methanolic extract of *C. militaris* in NCI-H460 cells") were washed in PBS and fixed in 4% paraformaldehyde (Merck) in PBS for 30 minutes at room temperature. Cells were then centrifuged and cell pellets were resuspended in PBS and kept at 4$^{o}$C until analysis. For analysis, cell cytospins were prepared by centrifuging at 500 rpm for 5 min in a cytospin centrifuge (Shandon). Cells were then incubated with 50 mM $NH_4Cl$ for 10 minutes, permeabilized with 0,2% Triton X-100 in PBS for 10 minutes and further incubated for 20 minutes in blocking buffer (2% BSA in PBS). Cells were incubated with rabbit anti-53BP1 antibody (1:200, Santa Cruz Biotechnology) at 4 $^{o}$C overnight, washed in 2% BSA (in PBS), and then incubated for 1 h with rabbit-IgG-FITC antibody (1:100; Dako). Slides were mounted with Vectashield mounting media with

DAPI (Vector laboratories) and images of the cells were taken (by Diana Sousa at IPATIMUP) using a fluorescence microscope (ZEISS Axio Imager.Z1) coupled with ApoTome Imaging System microscope. Exported images in TIFF format were decompressed with Irfanview (ver. 4.35, Irfan Skiljan, Vienna, Austria) and analyzed with ImageJ (version 1.46r, http://rsbweb.nih.gov/ij/index.html). This was carried out using a program written by Dr. Niklas Schultz (GMT Department, Stockholm University, Sweden (Markova *et al.* 2007)) which analyses DAPI and FITC channels independently, detects cell nuclei and registers foci parameters within the nuclear area. Different parameters, including the number of foci per nucleus were given in the output file.

## 3.4. Analysis of the effect of extracts from *Suillus luteus* and *Morchella esculenta* in THP-1 cell line

The effect of extracts from *Suillus luteus* and *Morchella esculenta* in THP-1 cell line were tested following two different protocols: i) MTS assay, for THP-1 monocytes and ii) Sulforhodamine B assay, for THP-1 differentiated macrophages.

### 3.4.1. MTS assay

For the MTS assay, the "CellTiter 96® AQueous One Solution Cell Proliferation Assay" (Promega) was used. THP-1 cells (50 µl) were plated at 1 x $10^4$ cell/well in 96-well plates (TPP-Techno Plastic Products) and treated with 50 µl of: complete medium (blank), five serial dilutions of each extract (ranging from 25 µg/mL to 400 µg/mL), or with maximum concentration of the vehicle tested ($H_2O$, control). Following 48 h incubation, MTS (20µl) was added to each well and incubated for 1-3 h at 37 $^oC$ in 5% $CO_2$. Absorbance was measured at 490 nm in a multiplate reader (BioTek® Synergy HT).

### 3.4.2. Sulforhodamine B assay (SRB)

Cells (100µl) were plated in 96-well plates (TPP-Techno Plastic Products) in cell culture medium with 20 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 18 h at 37°C in 5% $CO_2$, to induce differentiation. Cell culture medium was then replaced with fresh medium without PMA and cells incubated for 24 h to induce maturation (Roussaki *et al.* 2012). Cells were then treated with: medium only (100 µl) or with five serial dilutions of each extract (in duplicate) ranging from 25 µg/ml to 400 µg/ml. Another plate, called the " T0 plate" to which medium was added, was fixed at this point and further processed as explained below for the other plates. The remaining plates were incubated for 48 h, being then fixed by the addition of 50 µL ice-cold 10 % TCA (w/v) for 1 hour in ice and further washed 3 times with distilled water. After drying, plates were stained with 1% SRB in 1% (v/v) acetic acid and washed with 1% acetic acid. The bound SRB was solubilised in 10mM Tris-base solution (100µl) and the absorbance was measured at 510 nm in a multiplate reader (BioTek® Synergy HT).

## 3.5. Statistical analysis

For each assay, more than three independent experiments were carried out, unless otherwise stated in the results section. Statistical analysis was carried out using the Student's *t*-test. Differences were considered statistically significant whenever $P \leq 0.05$.

# IV

# RESULTS AND DISCUSSION

The present work was divided into two different parts: i) **Part I,** consisting in the study of the mechanism of action of *C. militaris* methanolic extract in the NCI-H460 cell line and ii) **Part II**, consisting in the study of the immunomodulatory activity of two extracts from different mushrooms, a polysacharidic extract from *Suillus luteus* and a phenolic extract from *Morchella esculenta*.

Therefore, the results will be presented and discussed in two different sections.

# Results - Part I

## 4.1. Study of the mechanism of action of *Cordyceps militaris* methanolic extract in the NCI-H460 cell line

The first part of the current project aimed at elucidating the mechanism of action of the methanolic extract of *C. militaris* (L.) Link fruiting body. As mentioned in the introduction, *C. militaris* has been regarded as having antitumor properties (Ng and Wang 2005). Several previous studies proved that this mushroom and their extracts have anticancer activity, with some compounds having been identified as contributors to this activity (e.g. cordycepin) (Paterson 2008, Liu *et al.* 2014). However, very few studies referred in particular to the antitumor potential of the methanolic extracts of *C. militaris*. Based on a previous study, from some of the collaborators of the research group, which showed that this extract presented antiproliferative potential in a panel of human tumor cell lines, including: MCF-7 (breast adenocarcinoma), HCT-15 (colon adenocarcinoma), HeLa (cervical adenocarcinoma) and NCI-H460 (NSCLC) (Reis *et al.* 2013), it was decided to further study its effect in the cell line in which it was most potent (NCI-H460). The choice of this cell line model had also into consideration the fact that it is representative of a type of cancer which is responsible for many deaths. In addition, this cell line has *wild-type* p53 which would allow detecting p53-dependent mechanisms of action.

### 4.1.1. Effect of *C. militaris* methanolic extract on NCI-H460 viable cell number

In order to confirm that the methanolic extract affected the viability of NCI-H460 cells, a preliminary experiment was carried out in which cells were treated for 48 h with *C. militaris* methanolic extract. To observe a possible dose/response effect, two concentrations were selected, 50 µg/mL [corresponding to the $GI_{50}$ concentration previously determined as being 47.8 µg/mL (Reis *et al.* 2013)] and 100 µg/mL [corresponding to twice the $GI_{50}$ ($2xGI_{50}$)]. Results, obtained after counting the number of viable cells with trypan blue (and analyzing results in relation to the number of viable cells in the blank, treated with medium only) showed a dose-dependent reduction in viable cell number, to 26.0% following treatment with 50 µg/mL and to 5.6% following treatment with 100 µg/mL (Figure 9).

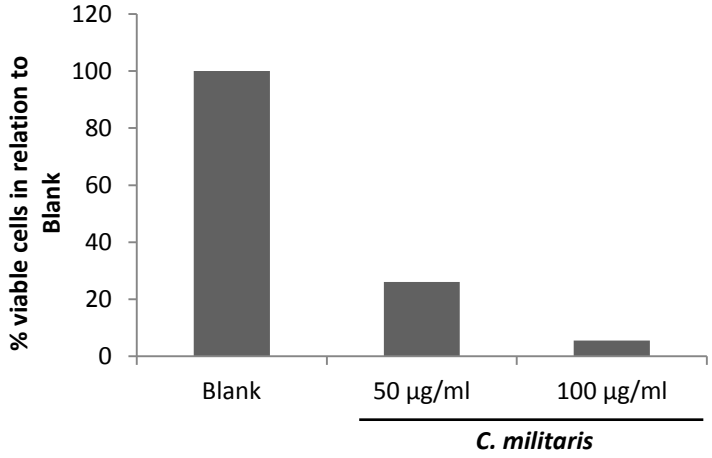


***Figure 9* –Effect of *C. militaris* methanolic extract in NCI-H460 viable cell number, analysed with Trypan Blue exclusion assay (Preliminary experiment).**

Cells were treated for 48 h with 50 µg/ml (corresponding to the $GI_{50}$ concentration) and 100 µg/ml (corresponding to the $2xGI_{50}$ concentration) of the extract. Results are presented as a percentage of viable cells in relation to blank cells (treated with medium only) and are from one experiment only.

These results showed that treatment with 50 µg/mL ($GI_{50}$) was causing a greater reduction of viable cell number than expected (since the $GI_{50}$ concentration reduces cell growth by 50%). The observed discrepancy between the $GI_{50}$ concentration previously determined by our collaborators with the sulforhodamine B (SRB) (Reis *et al.* 2013) and the viable cell number obtained with that same concentration is not abnormal in this type of studies and may be explained by the differences in the

methodologies used in both studies. Indeed, the SRB (which was carried out in the study by Reis *et al.* to determine the $GI_{50}$ concentration (Reis *et al.* 2013)) was carried out in 5% FBS culture medium, whereas all the assays carried out in the present study were performed in 10% FBS supplemented medium.

Based on the result of this preliminary experiment, it was decided to lower the concentration of extract and therefore analyze also the effect of 25 µg/mL (corresponding to ½ $GI_{50}$). Results showed a dose-dependent decrease in the percentage of viable cells to 45.8% and 31.1% following treatment with ½ $GI_{50}$ (25 µg/mL) and $GI_{50}$ (50 µg/mL), respectively (when compared to blank cells) (Figure 10). As expected, treatment with the solvent, water, at both concentrations [$H_2O$ (1) and $H_2O$ (2)], had no effect in NCI-H460 cells viability.

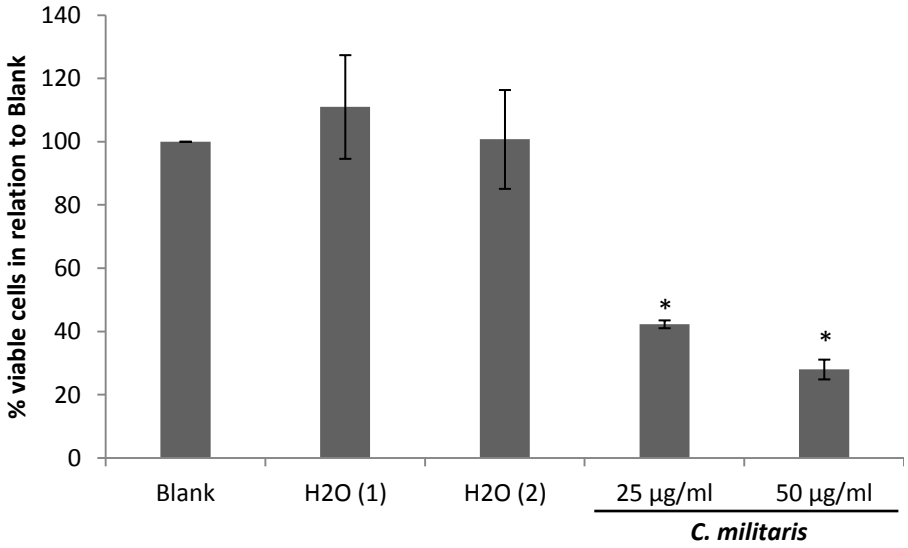


***Figure 10* - Effect of *C. militaris* methanolic extract in NCI-H460 viable cell number, analysed with Trypan Blue exclusion assay.**

Cells were treated for 48h with 25 µg/ml (corresponding to the ½ $Gi_{50}$ concentration) and 50 µg/ml ($GI_{50}$ concentration) of the extract; with the corresponding volumes of solvent [$H_20$ (1) and $H_2O$(2), respectively] or with medium only (Blank). Results are presented as percentage of viable cells in relation to blank cells and are the mean ± SEM of 6 independent experiments. *P ≤ 0.001 between each treatment and the Blank.

In order to further gain insight into the mechanism of action of the extract, these two concentrations of extract (25 µg/ml and 50 µg/ml) were used in the following experiments.

### 4.1.2. Effect of *C. militaris* methanolic extract on NCI-H460 cellular proliferation

To understand if the extract was affecting proliferation of NCI-H460 cells, BrdU incorporation was analysed by fluorescence microscopy, 48 h after treatment of cells with the extract. BrdU incorporation assay is based on the fact that cells can easily and specifically incorporate BrdU (a thymidine analog) into their newly synthesized DNA strands during cell proliferation (cells in S phase), which allows to monitorize DNA synthesis and cell proliferation. This incorporation can be easily detected, following partial denaturation of double stranded DNA, using FITC-labelled monoclonal antibodies against BrdU (Nagashima *et al.* 1985, Jayat and Ratinaud 1993, Moussa *et al.* 2005).

Results from this assay showed that the extract significantly decreased NCI-H460 cellular proliferation in a dose-dependent manner (Figure 11).

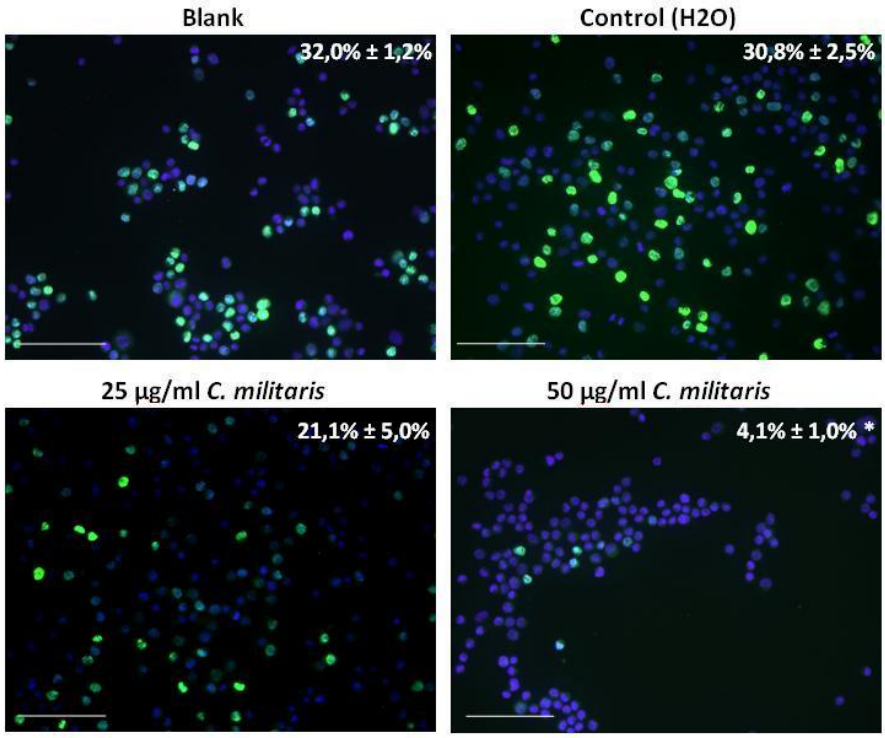


***Figure 11*- Effect of *C. militaris in* NCI-H460 cellular proliferation, analysed with the BrdU assay.**

Cells were treated for 48h with medium only (Blank), with the highest volume of $H_2O$ used (control), with 25µg/ml or 50µg/ml of *C. militaris* methanolic extract, or with. Proliferating cell nuclei are stained in green (from FITC labelling) and cell nuclei are stained in blue (from DAPI labelling). Immunofluorescence microscopy images are representative of three independent experiments.
*P ≤ 0.001 between each treatment and the Blank. Bar corresponds to 20 µm.

A decrease to 21.1% in the percentage of BrdU incorporating cells (compared to 32.0% in blank cells) was observed following treatment with 25 µg/ml of *C. militaris* methanolic extract. This decrease in the percentage of NCI-H460 cells undergoing proliferation was even more evident following treatment with 50 µg/ml, in which the levels of proliferation were 4.1%. As expected, control treatments with vehicle solvent had no effect on cellular proliferation.

### 4.1.3. Effect of *C. militaris* methanolic extract on NCI-H460 cell cycle profile

Given that a decrease in cellular proliferation might indicate that the extract affected the normal cell cycle progression, the cell cycle profile of NCI-H460 cells treated with the extract was analyzed by flow cytometry using propidium iodide (PI) labelling. This assay is based in the fact that PI binds stoichiometrically to nucleic acids. Since it is known that PI fluorescence in the cell is proportional to its content, it can be inferred that cells in G2/M phase have approximately twice the fluorescence of cells in G0/G1 phase (Nunez 2001). Flow cytometry analysis following PI labelling therefore allows for the measurement of cellular DNA content and the determination of cells distribution in the major cell-cycle phases (G1, S and G2/M) determined by peaks in a DNA content frequency histograms (Schönthal 2004).

Analysis of the cell cycle distribution of NCI-H460 cells following treatment with the extract showed clear alterations in their cell cycle profile, which were however only considered to be statistically significant with the highest concentration (50 µg/mL) (Figure 12). Indeed, treatment with 50 µg/ml of extract resulted in an increase in the percentage of cells in G0/G1 phase from 52.6% (Blank cells) to 71.7% following the treatment (Figure 12). This was accompanied by a decrease in the % of cells in both the S and the G2/M phases of the cell cycle. Indeed, a decrease in the S phase from 29.1% (Blank) to 17.1% (following treatment) was observed, as well as a decrease in the G2/M phase from 18.1% (Blank) to 7.0% (following treatment).

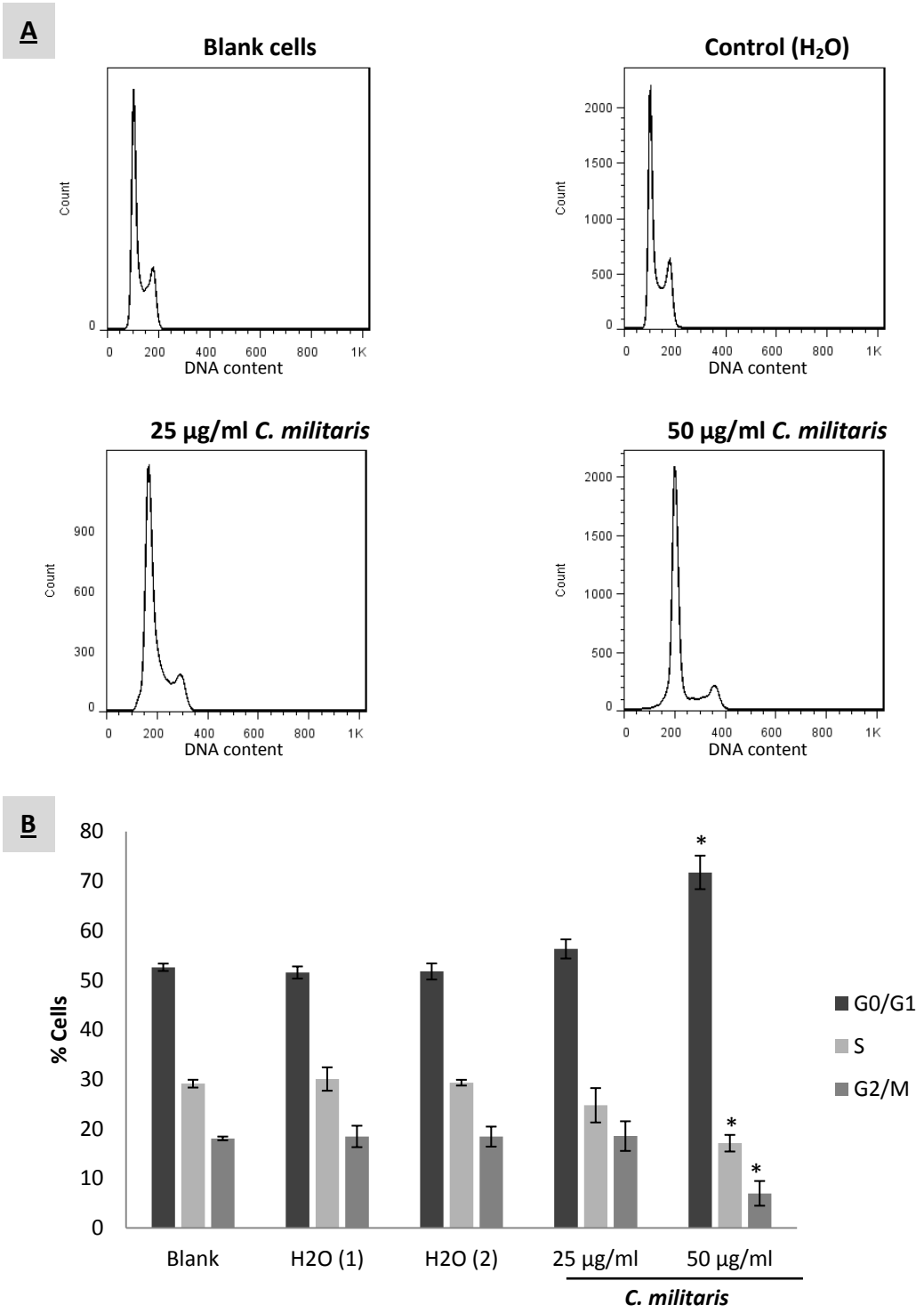


***Figure 12* - Effect of *C. militaris* methanolic extract in NCI-H460 cell cycle profile, analysed by flow cytometry.**

Cells, were treated for 48h with 25µg/ or 50µg/ml of *C. militaris* methanolic extract, with the corresponding volumes of solvent [H20 (1) and H2O (2), respectively and or medium only (Blank). **Panel A)** Histograms of the cell cycle profile. Control corresponds to the highest volume of $H_2O$. Images are representative of three independent experiments. **Panel B)** Cell cycle distribution. Results are the mean ± SEM of 3 independent experiments. *P ≤ 0.05 between each treatment and the Blank.

This effect was similar to the one described in a previous study for *C. militaris* and its mycelial fermentation. In that study, both fractions caused an arrest in G0/G1 phase of GBM8401 (Human brain malignant glioma) cells. Nevertheless, the same extracts induced an arrest in the G2/M phase of U-87MG cells, suggesting that the effect caused by the extract can be dependent of the genetic background of the treated tumor cells (Yang *et al.* 2012). Of interest is also the fact that cordycepin [an isolated compound from *Cordyceps* genus, which seems to be present in the methanolic extract from *C. militaris* (Liu *et al.* 2014)] induces cell cycle arrest in colorectal cancer cells at the G0/G1 phase (He *et al.* 2010). Although it is not known which compounds are responsible for the cell cycle arrest observed in the present study, the involvement of cordycepin in such activity is a possibility. Further studies, carried out in the cell line used in the present study could assess this hypothesis.

### 4.1.4. Effect of *C. militaris* methanolic extract on NCI-H460 cellular apoptosis

The decrease in cellular viability observed following treatment of NCI-H460 cells with the extract could also be due to increased apoptosis. This highly regulated energy-dependent process is important for the maintenance of tissue homeostasis and elimination of damaged cells, which involves specific morphological and biochemical mechanisms (such as chromatin condensation, reduction in cell volume, endonuclease cleavage of DNA into oligonucleosomal length fragments (Koopman *et al.* 1994, Elmore 2007, Park *et al.* 2009).

In order to assess if the *C. militaris* methanolic extract induced apoptosis in the NCI-H460 cell line, the levels of apoptosis were analyzed by flow cytometry following labeling with Annexin V-FITC/PI. This is a highly specific assay for apoptosis, based on the fact that during apoptosis some changes in the cell surface occur, namely the exposure of phosphatidylserine at the cell surface (being translocated from the inner side to the outer layer) as a result of the loss of membrane phospholipid asymmetry (Vermes *et al.* 1995). Therefore, the binding of Annexin-V-FITC to phosphatidylserine is used to detect apoptotic cells by flow cytometry (Koopman *et al.* 1994). In addition,

the combination of PI staining to the assay (which binds to nucleic acids only when the plasma membrane is compromised) allows for an indication of the cellular viability (Hawley and Hawley 2004, Riccardi and Nicoletti 2006).

By analyzing the levels of apoptosis in NCI-H460 cells, 48 h following treatment with the extract, it was possible to observe a clear increase in cellular apoptosis in both the concentrations tested (Table 4). An increase in the levels of apoptosis from 7.4 % (in Blank cells) to 17.1 % and 26.7 % was observed in NCI-H460 cells treated with 25 µg/mL or 50 µg/mL of the extract, respectively. These results showed that the extract was inducing apoptosis. As expected, and as previously observed in the other experiments carried out in this work, treatment with controls also had no effect on apoptosis.

**Table 4 - Apoptosis levels in NCI-H460 cells treated with *C. militaris* methanolic extract analyzed with Annexin V/PI staining by Flow Cytometry.**

| | | % Apoptosis |
|---|---|---|
| **Blank** | | 7.4 % ± 0.6 % |
| **$H_20$** | | 8,5 % ± 2.3 % |
| ***C. militaris*** | 25 µg/ml | 17.1 % ± 1.6 % * |
| | 50 µg/ml | 26.4 % ± 5.3 % * |

Cells were treated for 48h with 25 µg/ml or 50 µg/ml) of the extract, with the highest volume of solvent or with medium only (Blank). Results are the mean ± SEM of 3 independent experiments. *P ≤ 0.05 between each treatment and the Blank.

To further confirm these results, the levels of PARP cleavage were analyzed by Western blot. PARP is an enzyme that is cleaved by proteases activated during apoptosis, being a useful hallmark of apoptosis (Gobeil *et al.* 2001). Results, from two experiments only, showed a decrease in total PARP levels, which might indicate that PARP is being cleaved (Figure 13). However, the corresponding band for cleaved PARP could not be observed. This may be due to possible technical problems associated with the antibody used.

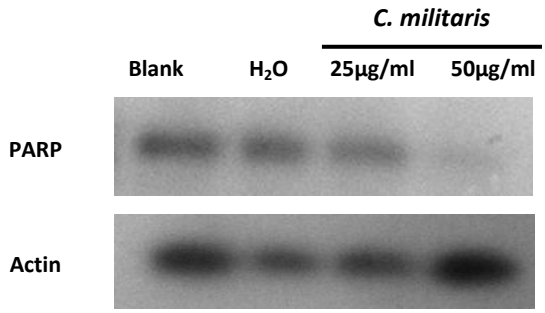


***Figure 13* - Protein expression of PARP in NCI-H460 cells treated with the methanolic extract of *C. militaris*, analyzed by Western Blot.**

Cells were treated for 48h with 25 µg/ml or 50 µg/ml of the extract, with the highest volume of solvent or with medium only (Blank). Actin was used as loading control. Image is representative of 2 independent experiments.

Several studies have previously referred the apoptotic activity of other types of extracts from *C. militaris*. Indeed, different aqueous extracts from *C. militaris* (AECM) were shown to induce apoptosis in human leukemia cells (Park *et al.* 2005, Lee *et al.* 2006), human breast cancer cells (Jin *et al.* 2008) and in human lung carcinoma cells (Park *et al.* 2009). In addition, some of the isolated compounds, namely Cordycepin, have shown activity as apoptosis inducers in several human tumor cells (Jeong *et al.* 2011; Baik *et al.* 2012; Tuli *et al.* 2013).

### 4.1.5. Effect of *C. militaris* methanolic extract on the cellular expression of p53 and p21

Since the previous results showed that the extract reduced proliferation, altered cell cycle profile and increased apoptosis in NCI-H460 cells, the levels of p53 and p21 were analyzed by Western blot.

Results showed an increase in the expression of p53 following cellular treatment with 25 µg/ml or 50 µg/ml of the methanolic extract of *C. militaris,* when compared to Blank and Control cells. This was further confirmed by the increased expression of p53's transcriptional target, p21 (Figure 14).

p53, the guardian of the genome, is a tumor suppressor gene which is activated when cells are under stress or damaged (Efeyan and Serrano 2007). p53 activation causes

blockage in cell cycle (and consequently in cellular proliferation) or may lead to apoptosis, thus allowing to prevent the development of the tumor (Amundson *et al.* 1998, Vogelstein *et al.* 2000). One of the downstream targets of p53 is p21 protein, an inhibitor of cyclin-dependent kinases (CDKs, which are regulators of the cell cycle). p21 inhibits CDKs and, consequently, inhibits the transition between G1 and S phases and between G2 and mitosis (Vogelstein *et al.* 2000). p53 is also known to induce apoptosis in damaged cells through activation of mitochondrial pro-apoptotic proteins from the Bcl-2 protein family, such as Bax, NOXA and P53AIP1 (Vogelstein *et al.* 2000). p53 is found mutated or inactivated in the majority of human cancers (Amundson *et al.* 1998, Vogelstein *et al.* 2000, Junttila and Evan 2009, Valente *et al.* 2013). Therefore, strategies aiming at the increase of p53's activity are of extreme importance for cancer therapy. NCI-H460 cells, the cells line used in this study, have wild-type (*wt*) p53. Nevertheless, even *wt* p53 cancers may also have downstream members of the p53 regulatory signalling pathway affected, which may lead to disruption in p53 functions (Athar *et al.* 2011). p53 activation and of its targets may result in p53-dependent increase in cell death and cell cycle arrest, inhibiting the development of *wt* p53 tumors.

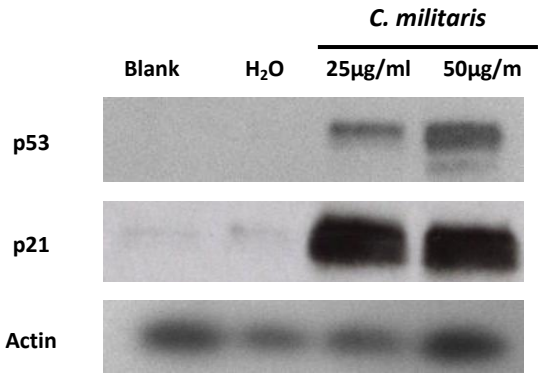


***Figure 14* – Expression levels of p53 and p21 expression in NCI-H460 cells following treatment with the methanolic extract of *C. militaris*, analyzed by Western Blot**

Cells were treated for 48h with 25 µg/ml or 50 µg/ml of the extract, with the highest volume of solvent or with medium only (Blank). Actin was used as loading control. Blot is representative of at least 3 independent experiments (except for the image of p53 in the 25 µg/ml treatment, which is representative of 2 experiments only).

### 4.1.6. Effect of *C. militaris* methanolic extract on DNA damage

Given that p53 activation may result from response to DNA damage (Amundson *et al.* 1998), the expression of P-H2AX was also analyzed in cells treated with the extract. H2AX, a histone which is only present in eukaryotes, is phosphorylated (into P-H2AX) when single stranded breaks (SSBs) and particularly double stranded breaks (DSBs) occur. Being the key factor in damaged DNA repair, P-H2AX may be used as a biomarker for DNA damage (Kuo and Yang 2008). Results showed an increase in the levels of P- H2AX following the treatment with both concentrations of the methanolic extract of *C. militaris* (Figure 15 and data not shown), further supporting the hypothesis that the extract induced DNA damage which then lead to an increase in p53 levels.

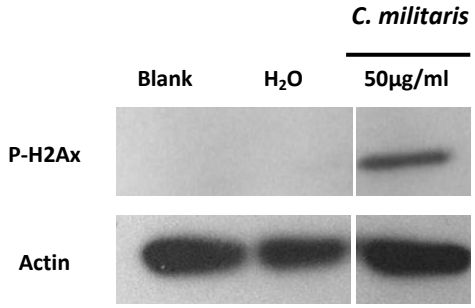


***Figure 15*- Expression levels of P-H2AX in NCI-H460 cells following treatment with *C. militaris* methanolic extract, analyzed by Western blot.**

Cells were treated for 48 h with 50µg/ml of the extract, with the corresponding volume of solvent or with medium only (Blank). Actin was used as loading control. Blot is representative of 2 independent experiments.

To further confirm DNA damage induced by the extract, the cellular localization of 53BP1 (p53-binding protein 1) was also analyzed by immunofluorescence. 53BP1 is a chromatin-associated factor described as being a DNA damage checkpoint mediator (DiTullio *et al.* 2002, Bartkova *et al.* 2007, Fradet-Turcotte *et al.* 2013). Following DNA damage, 53BP1 co-localizes with DNA damage response proteins, such as P-H2AX, playing a role early in the DNA damage response pathway (Ward *et al.* 2003, Kuo and Yang 2008). In addition, 53BP1 has also been proposed as a transcriptional co-activator of the p53 tumor suppressor (Schultz *et al.* 2000, DiTullio *et al.* 2002).

Results showed an increase in the number of small foci following treatment with both concentrations of *C. militaris* methanolic extract (Figure 16).

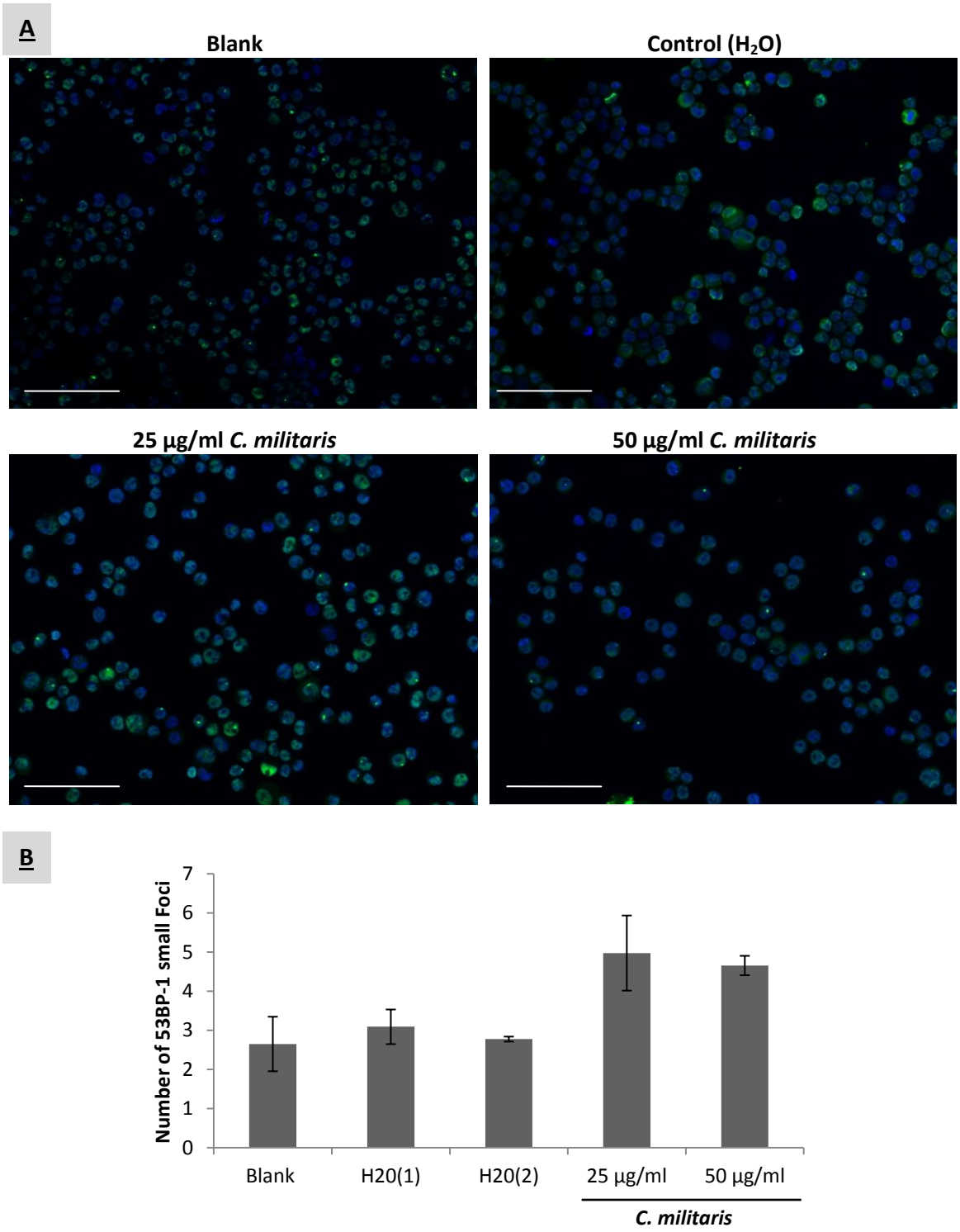


***Figure 16*- Effect of *C. militaris* methanolic extract in the levels of 53BP1 small foci in NCI-H460, analysed by immunoflourescence assay.**

Cells were treated for 48h with 25µg/ml or 50µg/ml of the extract, with the highest volume of solvent or with medium only (Blank). **Panel A)** Fluorescence microscopy images of 53BP1 staining in green (from FITC) and cell nuclei in blue (from DAPI). **Panel B)** Quantification of the number of 53BP-1 small foci. Results are represented as the mean ± SEM of 3 independent experiments. Iamges are representative of three independent experiments. Bar corresponds to 20 µm.

# Results - Part II

## 4.2. Effect of extracts from *Suillus luteus* and *Morchella esculenta* in THP-1 cell line

Modulation of the immune system and consequently of immune responses, aiming at helping the fight against diseases (such as cancer) has been of interest for the scientific and medical community (Sharififar *et al.* 2009).

This part of the project, aimed at studying the immunomodulatory effect of two mushrooms extracts (a polysacharidic extract from *S. luteus* and a phenolic extract from *M. esculenta*) in THP-1 cell line. This cell line is a suspension cell type which resembles circulating primary monocytes. When THP-1 cells are exposed to PMA, mitosis is stopped and monocytes adhere to the culture plate and differentiate into mature macrophages (Kang *et al.* 2014). Thus, this THP-1 cell line allows us to study the effect of mushroom extracts not only in monocytes but also in differentiated macrophages.

The work started by evaluating the effect of both extracts in the THP-1 cellular viability in order to exclude their possible cytotoxicity. This was carried out both in THP-1 monocytes as well as in THP-1 differentiated macrophages.

For this, two different assays were selected: i) MTS assay in the case of THP-1 monocytes and ii) Sulforhodamine B assay in the case of THP-1 macrophages. Since there is not much information in the literature about these methodologies when applied to this particular cell line, preliminary studies were carried out to optimize the corresponding protocols. The optimization procedure, as well as the preliminary results from the analysis of the cytotoxic effect of the extracts will be presented and discussed below.

### 4.2.1. Effect of a PLS extract from *S. luteus* and phenolic extract from *M. esculenta* in THP-1 monocytes

The MTS assay, a colorimetric assay that measures metabolic activity, was chosen to assess the effect of the extracts in THP-1 cells. This assay measures the production of aqueous soluble formazan following conversion of a tetrazolium salt by mitochondrial activity of viable cells at 37$^{o}$C. This coloured aqueous soluble formazan, produced by dehydrogenase enzymes, allows to indirectly determinate cell number, since the amount of formazan formed is directly proportional to the number of viable cells. When cells die, they lose the ability to convert MTS into formazan, thus color formation serves as a useful and convenient marker of viable cells only (Malich *et al.* 1997). The MTS is an alternative assay to the well known MTT assay, another colorimetric assay for assessing cell viability, with the advantage that the formazan formed from MTS is water soluble and less toxic when compared to the compound formed in the MTT assay (Wang *et al.* 2010).

The protocol for MTS assay applied to the THP1 cell line required optimization, in particular regarding the duration of incubation with MTS (prior to determination of absorbance), and the number of cells plated at the beginning of the assay. Therefore, in order to optimize the MTS protocol, increasing number of THP-1 cells were plated in different wells from a 96 well-plate and incubated for 48 h. After this period, the MTS solution was added to each well and the absorbance was measured at 1 h intervals, up to 4 h. Analysis of the results of formazan detection during the 4 h period of incubation with MTS, allowed to observe an increase in absorbance with the incubation period, with the maximum absorbance being measured at 3 h (Figure 17). No further increase in the absorbance was observed at 4 h. Therefore, the 3 h incubation period with MTS was chosen to analyse the results. When observing the absorbance obtained for the different number of cells plated, no differences were observed for the lowest range of cell number (below 2.500 cells/well). Indeed, clear differences were only observed in wells in which more than 5.000 cells were plated, increasing proportionally to the highest cell number analysed (12.500 cells/well) (Figure 17).

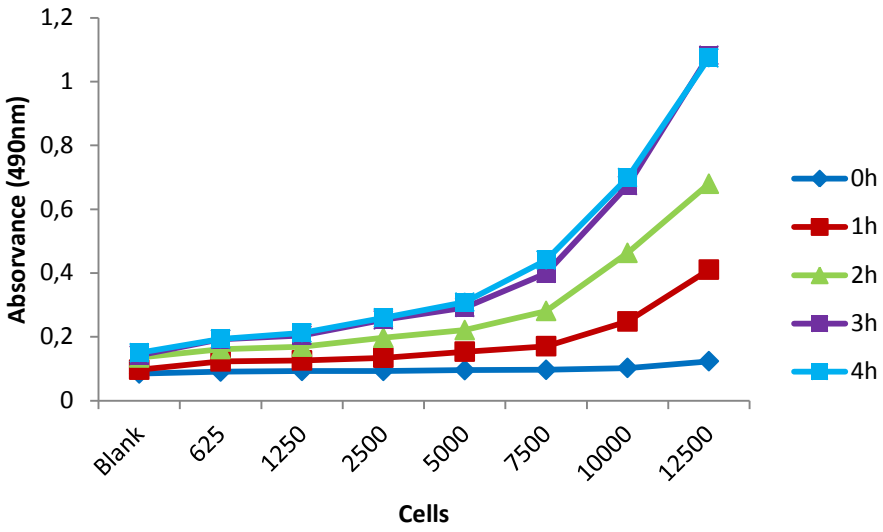


***Figure 17* - MTS Preliminary assay**

Cells were plated for 48 h. Absorvance was measured after MTS incubation for 4 h (at 1 h intervals). Results are from one experiment only.

Based on the results from this preliminary experiment, it was decided to carry out further experiments by plating cells at 10.000 cells/well for 48 h and by analysing absorbance following 3 h incubation with MTS.

In order to test the effect of the extracts in THP-1 monocytes, cells were treated for 48 h with five serial dilutions of each extract (PLS extract from *S. luteus* and phenolic extract from *M. esculenta*; both ranging from 25 µg/mL to 400 µg/mL).

Interestingly, results from the MTS assay showed a dose-dependent increase in metabolic activity of cells treated with PLS extract from *S. luteus* (Figure 18-panel A). Indeed, an increase to 150% and 180% was observed with the two highest concentrations of extract (200 µg/mL and 400 µg/mL, respectively). These results indicate that *S. Luteus* PLS extract not only is not toxic to THP-1 cells but also, and importantly, it seems to stimulate their proliferation, as suggested by the increase in MTS activity. When analysing the results following treatment with *M. esculenta* phenolic extract, no significant effect in metabolic activity of THP-1 cells was observed (Figure 18-panel B), which suggests that this extract is not cytotoxic to this cell line.

As expected, $H_2O$ (the extracts solvent) had no effect in cellular metabolic activity, and presented no toxicity to the cells.

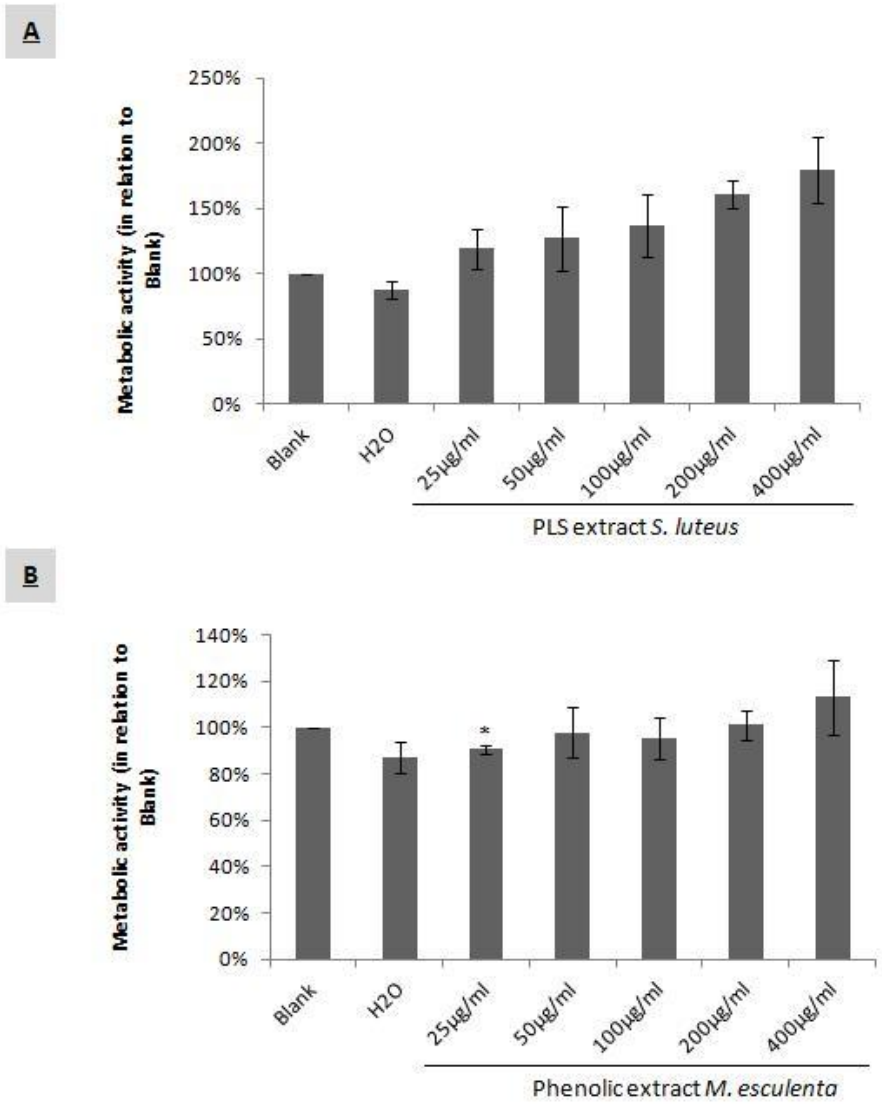


***Figure 18*** **- Effect of the extracts in the metabolic activity of THP-1 monocytes, analysed by MTS assay.**

Cells were treated for 48h with *S. luteus* PLS extract (**panel A**) and with *M. esculenta* phenolic extract (**panel B**). Absorbance was analyzed as percentage in relation to blank cells. Results are the mean ± SEM of 3 independent experiments. *P ≤ 0.05 between each treatment and the blank

Previous studies have shown that different extracts of *S. luteus* were not toxic to a non-tumor cell line model, namely primary cultures of porcine hepatocytes, at least in same range of concentrations as the ones tested in the present study (Santos *et al.* 2013). Although, there is no information regarding the cytotoxicity of *M. Esculenta.*

In addition to the evaluation of the effect of the extracts on THP-1 cellular viability (analysed with MTS assay) cells were carefully monitored in order to assess the possibility that the extracts induce their differentiation into macrophages. However, no alterations in the cellular morphology nor in the adhesion of the cells to the culture plates were observed during this period, indicating that neither of the extracts induce differentiation of the THP-1 cells.

### 4.2.2. Effect of PLS extract from *S. luteus* and phenolic extract from *M. esculenta* in THP-1 differentiated macrophages

To evaluate the effect of the same extracts in THP-1 differentiated macrophages (following treatment with PMA), the colorimetric SRB assay was carried out. SRB binds stoichiometrically to basic amino-acid residues under mild acidic conditions, and dissociates under basic conditions, thus allowing to indirectly determining cell density. (Vichai and Kirtikara 2006).

To optimize the SRB assay protocol for THP-1 differentiated macrophages, increasing number of cells were plated in 96 well plates, and treated with PMA for 18 h at 37 $^{o}$C to induce differentiation. After this period, cells were observed under an inverted optic microscope (Nikon Eclipse TS100) to confirm that cell differentiation (from monocytes to macrophages) had occurred. As previously referred (in the 4.2 section), this may be evaluated by the ability of adhesion gained by cells, following treatment with PMA. In fact, the cells observed had a more elongated, flat and amoeboid shape, suggesting that they had attached to the bottom of the plate. After confirming differentiation, cell culture medium was replaced with fresh medium (without PMA) and cells incubated for 24 h to induce maturation. After this period, fresh medium was added to cells which were further incubated for 48 h and then analysed with the SRB assay. The SRB assay was also carried out in the T0 plate (corresponding to the cells fixed at time zero - time at which the extracts were added) in order to assess cell growth following differentiation.

As expected, no differences were observed between cells in the T0 plate and cells following further 48 h in culture (Figure 19). This was already expected since it its known that differentiated cells do not proliferate (Qin 2012).

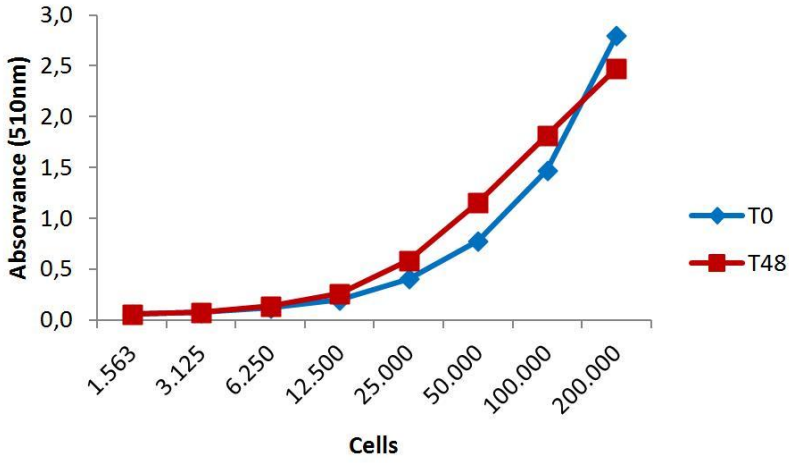


***Figure 19* - SRB Preliminary assay**

THP-1 differentiated macrophages were incubated for 48 h with medium. SRB assay was carried out at 0 h ()T0 and 48h (T48). Results are from one experiment only.

In addition, when analysing absorbance measured from the different number of cells plated, no differences were observed for the lowest number of cells plated (below 12.500 cells/well). Indeed, differences were only observed with more than 25.000 cells/well, increasing proportionally to the highest cell number analysed, 200.000 cells/well (Figure 19). Based on the results from this preliminary experiment, the following experiments were carried out starting with 50.000 cells/well.

To test the effect of the extracts in THP-1 differentiated macrophages, cells were treated for 48 h (after a period of differentiation and maturation as explained above) with five serial dilutions of each extract (PLS extract from *S. luteus* and Phenolic extract from *M. esculenta*; ranging from 25 µg/mL to 400 µg/mL).

Results from the SRB assay allowed to observe that both extracts, *S. luteus* PLS extract (Figure 20-panel A) and *M. esculenta* phenolic extract (Figure 20-panel B), had no significant effect in cell growth of THP-1 differentiated macrophages. This suggests that none of the extracts presented cytotoxicity to these cells. As previously mentioned, this had also been observed for THP-1 monocytes. Interestingly, the increased metabolic activity observed in THP-1 monocytes following treatment with *S.*

*luteus* PLS extract was not observed in the THP-1 differentiated cells. This may be explained by the fact that THP-1 monocytes correspond to an immature cell line, probably with more proliferation potential than the differentiated macrophages (a mature cell).

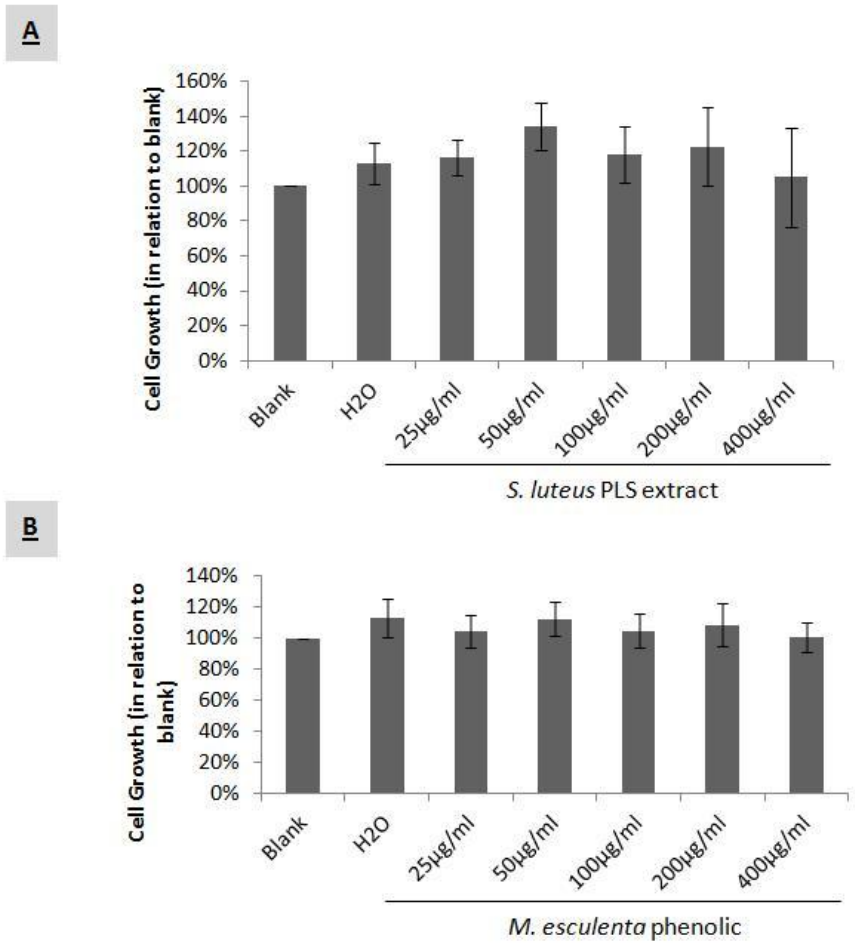


***Figure 20* - Effect of the extracts in THP-1 differentiated macrophages, analysed by SRB assay.**

THP-1 differentiated macrophages were treated for 48h with *S. luteus* PLS extract (panel A) or with *M. esculenta* phenolic extract (panel B). Absorbance was analyzed as percentage in relation to blank cells. Results are the mean ± SEM from 4 independent experiments.

Although the initial aim of this part of the work was to study the immunomodulatory effect of the referred extracts, it was not possible to completely achieve this goal, due to time constrains. Based on these results, future work will be carried out namely the immunomodulatory activity of *Suillus luteus* (polysachararidic (PLS) extract), *Morchella esculenta* (phenolic extract) and also other extracts from *Suillus luteus* and *Morchella esculenta* using the THP-1 cell line.

# V

# CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Mushrooms have been recognized not only for their nutritional but also for their medicinal properties. Indeed, a great interest has been given to exploring different bioactive compounds or extracts, which may have different properties. Despite the fact that there is intensive research on the therapeutic potential of several mushrooms, many of them, as well as their specific mechanisms of action, remain unknown (Rathee *et al.* 2012).

Therefore, this work aimed at further gaining insight into the antitumoral and immunomodulatory activity of different extracts from edible mushrooms.

In the first part of the study, the mechanism of action of a methanolic extract of *Cordyceps militaris* (which was previously shown to inhibitory the growth of human tumor cells) was investigated using NCI-H460 cells as a model. Results clearly showed that this extract decreased NCI-H460 viable cell number, causing a strong reduction in cellular proliferation, a cell cycle arrest in the G0/G1 phase and an increase in apoptosis. Moreover, treatment with this extract was shown to increase the expression levels of p53 and its transcriptional target p21, probably by the induction of DNA damage. Indeed, an increase in both the expression of P-H2AX and in the number of 53BP1 foci (both markers of DNA damage) were observed after treatment with this extract.

Although a few studies had already been carried out in human tumor cell lines using this extract, this was the first study in which its mechanism of action was associated to an increase in p53 expression. Since p53 is an important therapeutic target, due to fact that its activation may result in cell cycle delay and induction of apoptosis, extracts with proven ability to increase its expression may be of added value to the development of anticancer therapies. However, further studies would be needed in order to further explore this potential. In fact, it would be interesting to confirm if the same effect (observed in NCI-H460 cells) would be observed after silencing p53 expression (transiently with siRNAs or stably with shRNAs) in this cell line. One other approach to verify if the effects observed were p53-dependent would be to study the mechanism of action in other tumor cell lines which have different p53 status. Other studies suggest other possible therapeutic mechanisms caused by the *C. militaris* extracts and their compounds, e.g. associated with autophagy (Yang *et al.* 2012). It would be interesting to confirm this in future work. Also important would be to assess the specificity of this extract towards tumor cells, by testing this extract in non-tumor

cells and evaluating their possible toxicity. Regarding this, the study in which this work was based (Reis *et al.* 2013) had already described that this extract showed no effect in the cell growth of primary cells of porcine liver (PLP2). Nevertheless, it would be interesting to use human cell lines to address this issue. In addition, the effect of the extract could be also analysed in animal models (namely in models of human tumor cell lines xenografted in nude mice) to confirm its activity *in vivo.* Furthermore, it is not known if the observed effect is due to any particular compound included in the extract or the mixture of several compounds. The isolation and analysis of the effect of the different compounds present in this extract (including cordycepin) would be interesting to identify the bioactive compound(s) that is (are) responsible for the observed biological effects. Nevertheless, it is also possible that the whole extract presents an effect that is not reproducible by treatment with the isolated compounds.

In the second part of the study, an evaluation of the immunomodulatory potential of two different extracts, *S. Luteus* PLS and *M. esculenta* phenolic extract, was carried out in THP-1 cells. Although due to time constrains it was not possible to fully conclude this study, the work carried out allowed to analyse the effect of both extracts in the viability of THP-1 cell lines (both in monocytes as well as in macrophages). None of the extracts was found to be toxic towards THP-1 cells (monocytes and macrophages), at least in the range of concentrations analysed. Interestingly, an increase in THP-1 monocytes metabolic activity, suggestive of increased proliferation was observed after treatment. In addition, it was also possible to conclude that neither of extracts (at least in the range of concentrations analysed) induced differentiation of THP-1 monocytes into macrophages. Based on the information gathered, future studies specifically aiming at assessing the immunomodulatory activity of these compounds will be carried out by the research group. This may include analysis of the levels of mRNA and/or protein of NF-κB, pro-inflammatory cytokines (e.g. IL-1b and TNF-α) and inflammation-related enzymes (e.g. COX-2). If the immunomodulatory activity is proven, this may support the possible use of these extracts as an adjuvant to anticancer therapy.

Overall, the work carried out in this thesis further supports the potential of mushrooms extracts in the search for bioactive compounds.

# VI

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# VII

# ANNEXES

**Annex I:** Abstract Poster communication - IJUP 14 - 7th meeting Young Research of the University of Porto:

# Effect of *Cordyceps militaris* methanolic extract in NCI-H460 tumor cells

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Mushroom extracts have been studied extensively for their potential antitumor effect in tumor cell lines and in animal models [1]. Recently, some of our collaborators have described that the methanolic extract from the medicinal mushroom *Cordyceps militaris* (L.) Link presented tumor cell growth inhibitory activity. In particular, this extract inhibited the growth of the non-small cell lung cancer (NSCLC) cell line NCI-H460, presenting a $GI_{50}$ of approximately 50 µg/ml [2].

The aim of this work was to further study the effect of *C. militaris* methanolic extract in NCI-H460 cells, regarding its mechanism of action.

NCI-H460 cells were treated with *C. militaris* methanolic extract (at 25 and 50 µg/ml) for 48 h. Viable cell number was then assessed with the Trypan blue exclusion assay. Cellular proliferation was analyzed with the BrdU incorporation assay and cell cycle profile with flow cytometry following propidium iodide (PI) labeling. Levels of apoptotic cell death were determined with flow cytometry following Annexin V-FITC /PI labeling.

Treatment of cells with the *C. militaris* extract caused a dose-dependent decrease in viable cell number. Moreover, a clear and strong decrease in cellular proliferation was observed. In addition, alterations in cell cycle profile were found, with a strong decrease in the S and G2/M phases of the cell cycle together with an increase in G0/G1 phase. Furthermore, treatment with the extract also induced apoptosis in this cell line.

In conclusion, *C. militaris* methanolic extract was shown to interfere with cell proliferation, cell cycle and to induce apoptosis of NCI-H460 cells. Further studies will aim at further understanding the mechanism of action of this extract.

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# Effect of *Cordyceps militaris* methanolic extract in NCI-H460 tumor cells

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Porto, February, 12-14, 2014

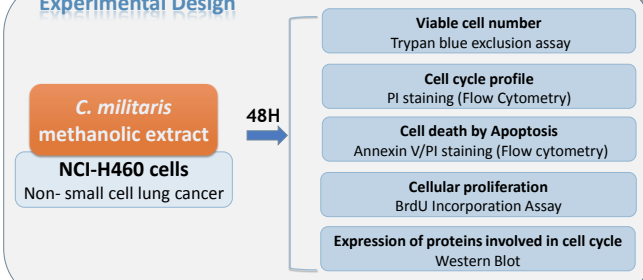
## Background

Mushroom extracts have been studied extensively for their potential antitumor effect in tumor cell lines and in animal models [1]. Recently, some of our collaborators have described that the methanolic extract from the medicinal mushroom *Cordyceps militaris* (L.) Link presented tumor cell growth inhibitory activity. In particular, this extract inhibited the growth of the non-small cell lung cancer (NSCLC) cell line NCI-H460, presenting a $GI_{50}$ of approximately 50 µg/ml [2].

## Aim

To further study the effect of *C. militaris* methanolic extract in NCI-H460 cells, regarding its mechanism of action.

## Experimental Design




## Results

### Effect of *C. militaris* methanolic extract on NCI-H460 viable cell number

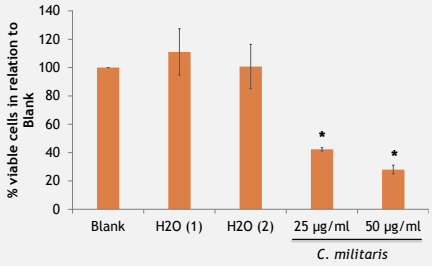



**Figure 1. Analysis of the effect of *C. militaris* methanolic extract in NCI-H460 viable cell number with Trypan Blue exclusion assay**. Cells were treated for 48 h with 25 µg/ml (corresponding to the ½ Gi50 concentration) and 50 µg/ml (corresponding to the Gi50 concentration) of *C. militaris* methanolic extract, with the corresponding volumes of solvent [$H_2O$ (1) and $H_2O$ (2), respectively]. Results are presented as a percentage of viable cells in relation to blank cells (treated with medium only) and are shown as mean ± SE of 3 independent experiments. * P ≤ 0,05 between each treatment and the Blank.

➢ ***C. militaris* methanolic extract decreased NCI-H460 cells viability**

### Effect of *C. militaris* methanolic extract on NCI-H460 cellular proliferation

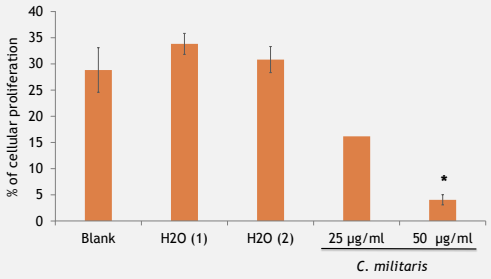



**Figure 3. Analysis of the effect *C. militaris* methanolic extract in NCI-H460 cellular proliferation by the BrdU incorporation assay.** Results are presented as a percentage of cellular proliferation and are shown as mean ±SE of 3 independent experiments (except for the case of 25 µg/ml in which the results are the mean of 2 experiments only). * P ≤ 0,05 between each treatment and the Blank.

➢ ***C. militaris* methanolic extract decreased NCI-H460 cellular proliferation**

### Effect of *C. militaris* methanolic extract on NCI-H460 cell cycle profile

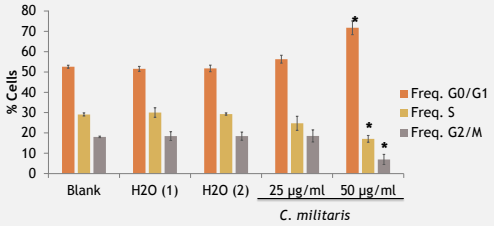



**Figure 2. Analysis of the effect of *C. militaris* methanolic extract in NCI-H460 cell cycle profile by flow cytometry.** Results are the mean ± SE of 3 independent experiments. * P ≤ 0,05 between each treatment and the Black.

➢ ***C. militaris* methanolic extract affected the NCI-H460 normal cell cycle profile:**
- **Decrease in S and G2/M phases**
- **Increase in G0/G1 phase**

### Effect of *C. militaris* methanolic extract on NCI-H460 cellular apoptosis

**Table 1. Percentage of apoptotic cells, analysed with Annexin V/PI staining by Flow Cytometry**

| | | % Apoptosis |
|---|---|---|
| Blank | | 6.3% ± 1,4% |
| $H_2O$ (1) | | 5,8% ± 1.6% |
| $H_2O$ (2) | | 7.1% ± 2,1% |
| *C. militaris* | 25 µg/ml | 14.2% ± 3,1%* |
| | 50 µg/ml | 23.0% ± 5,2%* |

Results are presented as a percentage of apoptotic cells and are shown as mean ±SE of 4 independent experiments. * P ≤ 0,05 between each treatment and the Blank.

➢ ***C. militaris* methanolic extract induced apoptosis in NCI-H460 cells**

### Effect of *C. militaris* methanolic extract on the cellular expression of p53 and p21

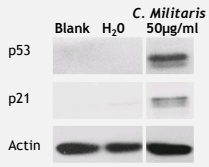



**Figure 4. Expression of p53 and p21 in NCI-H460 cells following treatment with 50µg/ml *C. militaris*, analysed by Western blot**. Actin was used as loading control. Blot is representative of 2 or 3 independent experiments (for p21 and p53 respectively).

➢ ***C. militaris* methanolic treatment increased the expression of p53 and p21 in NCI-H460 cells**

## Conclusion

*C. militaris* methanolic extract was shown to interfere with cellular proliferation, cell cycle profile and to induce apoptosis of NCI-H460 cells. Further studies will aim at further understanding the mechanism of action of this extract.

## References

[1] Ferreira *et al*. Anti-cancer agents in medicinal chemistry.2010; 10:424-436

[2] Reis *et al*. Food and chemical toxicology, 2013; 62:91-98.