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Effect of mushroom polysaccharides and olive phenolic compounds on human carcinoma cells

Diana Jorge Machado de Figueiredo da Fonseca Martinho

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Dedicatória:

Dedico esta tese de mestrado aos meus **Pais e irmã**,
Cuja paciência, amor e carinho me ajudaram a superar todas as dificuldades

Às minhas avós, por serem modelos de coragem e bondade a seguir.
"Tu podes, assim tu queiras"

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Resumo

Hoje em dia, devido ao prolongamento da esperança média de vida, bem como o aumento das agressões sofridas pelo meio ambiente envolvente, o cancro é uma das doenças mais proeminentes no seculo XXI.

Neoplasmas, conjuntos anormais de células cromossomicamente mutadas, levam ao crescimento relativamente autónomo de tecido danificado. As causas destas mutações variam entre internas - condições imunitárias, hormonais, heritabilidade - ou externas - como tabagismo, exposição a radiação ou agentes químicos infecciosos (Zaidman *et al.*, 2005).

As células neoplásicas caracterizam-se pela perda das suas funções especializadas e ganham por sua vez características biológicas tais como a produção de sinais de crescimento, inibição da apoptose, elevado potencial replicativo e a capacidade de invadir tecidos circundantes (Zaidman *et al.*, 2005; Zong *et al.*,, 2012). A esta metastização dos tecidos está associada 90% da responsabilidade da morte por cancro (Zong *et al.*,, 2012).

Apesar da eficiência dos fármacos gerados até hoje no combate ao cancro, o seu efeito tóxico causa na maioria dos casos um decréscimo acentuado na qualidade de vida dos doentes. Assim, torna-se importante descobrir novos agentes anti cancerigenos com efeitos tóxicos mais baixos (Cao *et al.*, 2010) que possam ser utilizados em terapias complementares.

Virámo-nos então de novo para as tradições mais antigas da medicina Chinesa e Mediterrânea.

Polissacáridos são biopolímeros constituídos por várias unidades de monossacáridos ligados através de ligações glicosidícas, que podem apresentar diferentes conformações, não só nas suas unidades constituintes, mas também na conformação das suas ligações, atribuindo-lhes assim diversas funções biológicas. Estes estão presentes desde o armazenamento de energia (amido), formação de componentes estruturais (celulose), participantes na comunicação célula a célula, possuindo ainda papéis importantes no sistema imunitário, na fertilização, desenvolvimento, agregação plaquetária e prevenção de patogéneses (Zong *et al.*, 2012).

Desde a medicina ancestral chinesa que os cogumelos têm sido reportados como tendo propriedades imunomoduladoras - actuando como modificadores da resposta biológica - e possuindo propriedades antivirais, antimicrobiais, circulatórias e nas ultimas décadas anti cancerigenas. (Wasser, 2010). A descoberta do Lentinian, um polissacárido com propriedades anti cancerígenas, despoletou nos anos 60 a pesquisa que tem sido dedicada ate hoje ao campo dos polissacáridos (Cao *et al.,* 2010).

Ao longo do tempo, diversos polissacáridos produzidos por cogumelos, têm demonstrado largos efeitos inibidores em vários tipos de tumores como Sarcoma 180, carcinoma de Lewis e Yoshida Sarcoma (Zhang *et al.,* 2007).

Existem três mecanismos propostos de actuação destes polissacáridos: (1) actividade preventiva, (2) aumento da resposta imunitária do hospedeiro e (3) inibição directa quando em contacto com células cancerígenas (Zhang *et al.,* 2007).

Na sua maioria os cogumelos que possuem polissacáridos biologicamente activos pertencem ao grupo dos *basidiomicetes* contendo cerca de 700 espécies descritas (Wasser, 2002).

O seu poder antioxidante também desempenha um papel fundamental contra o desenvolvimento cancerígeno. Anti oxidantes naturais encontrados em fruta, vegetais, cogumelos e outros recursos alimentares têm sido investigados para prevenção de doenças coronárias e outras como o cancro (Kozarski *et al.,*2012).

Assim, na procura de compostos não tóxicos que possuíssem propriedades anticancerígenas investigou-se também um pouco dos efeitos que as folhas de oliveira possam ter no combate ao cancro.

A oliveira (*Olea europaea*) é uma árvore da família das Oleaceae, (Zaid *et al.,* 2012) com folhas alongadas, que se encontra ao longo de toda região Mediterrânea desde há mais de 7000 anos (Lalas *et al.,* 2011).

Ao longo da história as folhas de *Olea europaea* têm sido exploradas para a prevenção de hipertensão, carcinogénese, diabetes, arteroesclerose bem como outras doenças do foro mais comum (Bouallagui *et al.,* 2011; Zaid *et al.,* 2012). A folha de oliveira foi ainda utilizada ao longo da história como um meio de combate á malária (Lee *et al.,* 2009)

A incidência de cancro ao longo do mediterrâneo tem sido das mais baixas quando comparada com outras partes do mundo, principalmente quando nos referimos ao cancro da mama, do endométrio, próstata e leucemia. (Bouallagui *et al.,* 2011; Zaid *et al.,* 2012)

Assim reunimos neste trabalho, o estudo dos efeitos de algumas espécies conhecidas (e outras menos conhecidas) de cogumelos, com o estudo dos efeitos de algumas variedades de oliveiras nacionais, na inibição do crescimento de células tumorais.

Para isso, foram efectuados vários doseamentos de polissacáridos, proteínas, açucares e compostos fenólicos e foram aplicadas diversas amostras das espécies amostradas a quatro linhas de carcinoma humano: HeLa, carcinoma cervical, A549 carcinoma pulmonar; A431 carcinoma epidermóide e OE21 carcinoma esófago-faringeal.

De entre os variados resultados obtidos, pudemos concluir que a aplicação de extra polissacáridos da estirpe *Ganoderma carnosum* revelou um alto teor inibitório, e que os intra polissacáridos de todas as estirpes amostradas mostraram uma capacidade anti-oxidativa acima de 50%.

As folhas de oliveira por sua vez, foram também capazes de mostrar um potencial anti proliferativo das células tumorais bastante satisfatório.

Acreditamos que nestes compostos podem estar presentes as condições para a sua aplicação como terapia complementar às terapias anticancerígenas existentes.

Palavras chave: anti cancerígeno, polissacáridos; cogumelos, compostos fenólicos, oliveiras, linhas de carcinoma humano.

Abstract

Cancer is widely known nowadays as the disease of the century. Not only by the number of infected people worldwide but also by the amount of research dedicated to this field and its increasing success rate.

Neoplasms, which are abnormal masses or colonies of cells produced by a relatively autonomous new growth of tissue, arise normally by the clonal expansion of a single cell that has undergone mutation in the chromosomal DNA, caused by a chemical, physical or biological agent (Zaidman *et al.*, 2005). One of the most characterizing features of cancers, besides it's ability to resist treatment, it's his ability to metastasize and invade surrounding tissues, causing 90% of cancer deaths (Zong *et al.*, 2012)

Although the efficiency of chemotherapy for the majority of cancers has improved over the last three decades, the drugs used in this treatment, contain high toxic effects that cause severe reduction in quality of life. Therefore, it is important to develop novel potent, but low toxic anti-cancer reagents, including natural products (Cao *et al.*, 2010).

The desire to find this new chemical drug as lead us to look back in time and to report to times of ancient Chinese and Mediterranean medicine, in order to encounter natural products that can in the future be developed as efficient co-chemotherapeutical agents.

During this work we will test mushroom polysaccharides as well as olive leaves phenolic compounds, in human carcinoma cell lines, in order to discover more about their anti-tumor effects.

Amongst our findings, extrapolysaccharides from *Ganoderma lucidum* were the most promising and all intrapolysaccharides were found to have a 50% rate of anti-oxidative power.

Olive leaves were also very promising in the inibithion of human carcinoma cells *in vitro.*

Key Words: anti-cancer; polysaccharides, mushrooms; phenolic compounds; olive human carcinoma cell lines;

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1 State of the art

1.1 What is Cancer?

Cancer is widely known nowadays as the disease of the century. Not only by the number of infected people worldwide but also by the amount of research dedicated to this field and its increasing success rate.

Neoplasms, which are abnormal masses or colonies of cells produced by a relatively autonomous new growth of tissue, arise normally by the clonal expansion of a single cell that has undergone mutation in the chromosomal DNA, caused by a chemical, physical or biological agent, (Zaidman *et al.,* 2005) both external (tobacco, alcohol, chemicals, infectious agents and radiation) and internal (hormones, immune conditions, inherited mutations, and mutations occurring in metabolism) that directly and irreversibly alter the cell genome. (Zong *et al.,* 2012)

Neoplastic cells are characterized by the loss of some specialized functions and acquisition of new biological proprieties, like self-sufficient growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, invasion of surrounding tissues, metastasis to other tissues and organs and finally the passage of those biological proprieties to progeny cells (Zaidman *et al.*, 2005; Zong *et al.*, 2012).

This can further be clinically considered benign or malignant.

Cancer is a generic term used to describe malignant neoplasms which manifest a greater degree of autonomy, capability of metathesized expansion and capacity to resist treatment and cause death. Anaplasia is a representative propriety and denotes a lack of normal structural and functional cell characteristics (Zaidman *et al.,* 2005).

The ability to invade and metastasize is the defining characteristics of a cancer. After the transformation from a normal cell into a malignant cell via genetic mutation, cancerous cells proliferate rapidly, break off from the parent lump, migrate around the body in the blood or the lymphatic system, and set up secondary foci of cancerous

growths at distant sites. Metastasis is responsible for 90% of the cancer death rate (Zong *et al.*, 2012).

A report released by the World Health Organization (WHO) has showed that an estimated 12.7 million people globally were diagnosed with cancer and about 7.6 million people died of it in 2008. As estimated in this report, more than 21 million new cancer cases and 13 million deaths are expected by 2030 (Zong *et al.*, 2012).

Although the efficiency of chemotherapy for the majority of cancers has improved over the last three decades, the drugs used in this treatment, while formidable, contain high toxic effects that cause severe reduction in quality of life, causing problems in clinical medicine. Therefore, it is important to develop novel potent, but low toxic anti-cancer reagents, including natural products (Cao *et al.*, 2010).

1.2 Polysaccharides

Polysaccharides are biopolymers comprised of monosaccharides linked together through glycosidic bonds. These structures can be linear or contain branched side chains. Polysaccharides have a general formula of C_x $(H_2O)_y$ where x is usually a large number between 200 and 2500. Considering that the repeating units in the polymer backbone are often six-carbon monosaccharides, the general formula can also be represented as $(C_6H_{10}O_5)$ n where $40 \leq n \leq 3000$ (Zong *et al.*, 2012).

Polysaccharides can be classified into two groups based on their source. Natural polysaccharides are obtained from several organisms such as algae, plants, microorganisms or animals and semi-synthetic polysaccharides, produced by chemical or enzymatic modification of the parent macromolecules. Polysaccharides obtained from different sources and by different chemical manipulations exist in a variety of chemical compositions, molecular weights and structures. They possess several physic - chemical properties including gelation, solubility, low osmotic effect and surface properties depending on their composition and architecture (Zong *et al.*, 2012).

Since they are a structurally diverse class of macromolecules, polysaccharides play diverse and important roles in many biological processes. As well as serving as stores of energy (e.g. starch and glycogen) and structural components (e.g. cellulose in plants and

chitin in arthropods), polysaccharides and their derivatives can participate in signal recognition, cell – cell communication and also play key roles in the immune system, fertilization, pathogenesis prevention, blood clotting and system development (Zong *et al.*, 2012).

Recently, oligosaccharides have demonstrated a broad spectrum of biological effects, such as antibiotic, antioxidant, anti-mutant, anticoagulant and immuno-stimulation activities. The anti-cancer efficiency of polysaccharides was first recognized by Nauts *et al.*, in 1946 when she discovered that certain polysaccharides could induce complete remission in patients with cancer (Zong *et al.*, 2012).

1.2.1 Mushroom polysaccharides

Since the discovery that Lentinan, a polysaccharide from *Lentinula edodes*, inhibited mouse sarcoma 180 and displayed very low toxicity (when compared with chemical antitumor drugs) a number of polysaccharides with antitumor activity have been reported (Cao *et al.*, 2010). This led to new scientific and medical studies, increasingly demonstrating the medicinal proprieties of mushroom-extracted compounds for the prevention and treatment of cancer (Zaidman *et al.*, 2005).

It has been well established that many mushroom-extracted compounds, like polysaccharides, are commonly used as immunomodulators or biological response modifiers (BRM) (Zaidman *et al.*, 2005; Wasser, 2010; Liang *et al.*, 2011) ie, they cause no harm and place no additional stress on the body, but help it to adapt to environmental and biological stress, being mainly used as an adjuvant therapy approach in clinical trials (Sarangi *et al.*, 2006; Liang *et al.*, 2011)

In literature, a "mushroom" is: a macro-fungus with a distinctive fruiting body that is large enough to be seen by the naked eye and to be picked up by hand and commonly occurs in two classes: *Basidiomycetes* and ,sometimes, *Ascomycetes* (Zhang *et al.*, 2007). Figure 1 represents there life cycle, including the structures where polysaccharides can be found.

Many higher *Basidiomycetes* mushrooms contain biologically active polysaccharides in its fruit bodies, cultured mycelium and cultured broth (Wasser,

2002). Data on mushroom polysaccharides is summarized for approximately 700 species of higher Hetero - and Homobasidiomycetes (Sarangi *et al.*, 2006;Wasser, 2010; Wieter *et al.*, 2011).

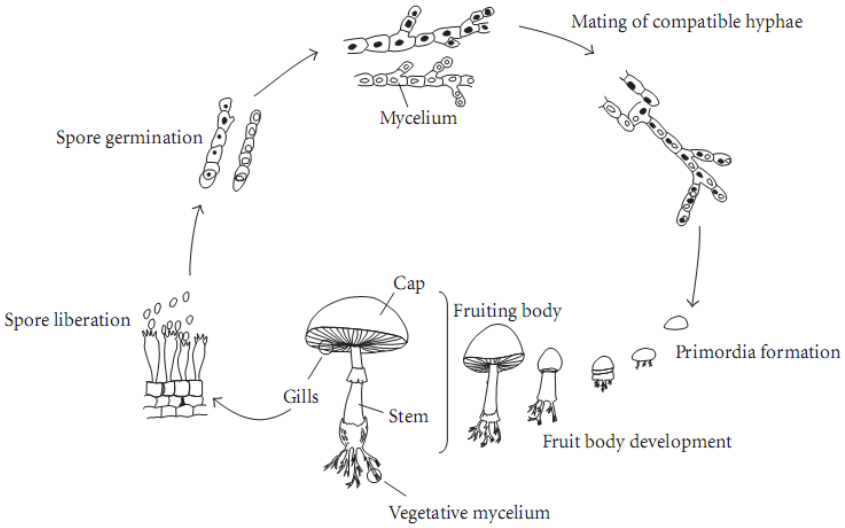


Figure 1 - Diagrammatic representation of mushroom life cycle (Lull *et al.*, 2005)

Mushrooms have been part of a normal human diet for thousands of years and, in the last decade, the amounts consumed have risen greatly, involving a large number of species (Jayakumar *et al.*, 2011).

In general, the total composition of mushrooms is water (90%), protein (2-40%), carbohydrates (1-55%), fiber (3-32%) and ash (8-10%). The ash is made up mainly of salts and metals like calcium and magnesium (Ishii *et al.*, 2011). They are valuable health foods since they are low in calories, fats, and essential fatty acids, and high in vegetable proteins, vitamins and minerals. Mushrooms are the only non-animal-based food containing vitamin D (Jayakumar *et al.*, 2011).

For millennia, medicinal mushrooms have established a history of use in traditional ancient therapies and contemporary research has validated and documented much of this ancient knowledge. The interdisciplinary field of science that studies medicinal mushrooms has been increasingly developing and demonstrating the potent and unique properties of compounds extracted from a range of mushroom species (Wasser, 2010).

Figure 2 - *Pleurotus ostreatus* and *Pleurotus eryngii*
(http://www.morelmushroomhunting.com/pleurotus_castreatus.htm)

Figure 3 –: *Irpex lacteus* (www.hoosiermushrooms.com/blog/) and *Bjerkandera adusta*
(www.herbarium.iastate.edu/fungi/fungispecies.php?sp=Bjerkandera+adusta+%28Willd.%3AFr.%29+Karst.)

Numerous bioactive polysaccharides or polysaccharide – protein complexes from medicinal mushrooms are described as a manner to enhance innate and cell-mediated immune responses and exhibit antitumor activities in animals and humans (Wasser, 2010).

The stimulation of immune defense systems has significant effects on the maturation, differentiation and proliferation of many kinds of immune cells in the host. Many of these mushroom polymers were reported to have immunotherapeutic properties by facilitating growth inhibition and destruction of tumor cells (Wasser, 2010).

Recently, some of the medicinal effects associated to mushrooms included antitumor, immunomodulating, antioxidant, radical scavenging, cardiovascular, antihypercholesterolemia, antiviral, antibacterial, antiparasitic, antifungal,

detoxification, hepatoprotective and antidiabetic effects. Mushroom polysaccharides can prevent oncogenesis, show direct antitumor activity against several synergetic tumors and prevent tumor metastasis, being especially beneficial when used in conjunction with chemotherapy Although the mechanism of their antitumor actions is still not completely understood, stimulation and modulation of key host immune responses by these mushroom polymers appears to be central (Carbonero *et al.*, 2008; Wasser, 2010).

Figure 4 - *Ganoderma apllanatum* (http://www.first-nature.com/fungi/ganoderma-applanatum.php)

Thus, the benefits of mushroom compounds on different clinical conditions have attracted the interest of the scientific community in the last decade in order to understand the molecular mechanisms responsible for their actions. Several classes of mushroom compounds such as proteins, polysaccharides, lipopolysaccharides and glucoproteins have been classified as molecules with potent effects on the immune system that may restore and enhance immunological responses of host immune effector cells (Wasser, 2010).

Polysaccharides or extracts mainly containing polysaccharides from more than 30 medicinal mushrooms species have shown anti-cancer activity in animal models, but only a few of them have been further taken to clinical assessment in humans (Wasser, 2010; Zong *et al.*, 2012).

1.2.2 Structural and physical properties

A wide range of antitumor or immune-stimulating polysaccharides of different chemical structures have been investigated. Polysaccharides with strong anti-tumor action may differ greatly in their chemical structures. Antitumor activity is exhibited by a wide range of glycans extending from homopolymers to highly complex heteropolymers (Zhang *et al.*, 2007; Yang & Zang, 2009; Synytsya *et al.*, 2009).

A large group of bioactive polysaccharides is called heteroglycans which are classified as galactans, fucans, xylans and mannans depending on the individual sugar components in the backbone. Heteroglycan side chains may contain arabinose, mannose, fucose, galactose, xylose, glucuronic acid and a glucose moiety as a main component, or in various combinations (Zhang *et al.*, 2007; Yang & Zhang, 2009).

The structure of naturally occurring glycans is so diversified that it is difficult to define a universal protocol for their analysis. The building blocks (derived from sugar residues) of complex polysaccharides exhibit very similar structures, but their differentiation (diversified linkage style) is widely varied (Zhang *et al.*, 2007). The primary structure of a polysaccharide is defined by monosaccharide composition and sequence, configuration and position of glycosidic linkages as well as the nature, number and location of appended non-carbohydrate groups (Zhang *et al.*, 2007).

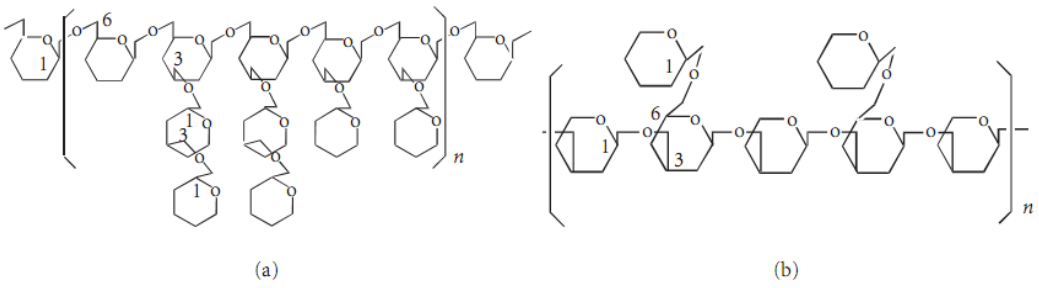


Figure 5 - Repeating unit of immunomodulatory β-glucans (a) from *Grifola frondosa* and (b) from *Lentinula edodes* (Lull *et al.*, 2005)

Polysaccharides are usually composed of several monosaccharides linked with different glycosidic bonds. Some have hyper-branched structures, high molecular weights and tend to form aggregates in solution that may mask the behavior of individual micro molecules (Yang & Zhang, 2009).

In some mushroom species, polysaccharides are bound with proteins or peptides to form complexes which show higher potent antitumor activity (Zhang *et al.,* 2007).

The major component in the biologically active polysaccharides was identified as β-D-glucans (Wong *et al.,* 2011). The anti-tumor activities may be related to the triple helical conformation of the (1-3) – β-D-glucan backbone chain for some, such as lentinan from *Lentinus edodes* (Yang & Zhang, 2009). The few human tested polysaccharides are rich in β-D-glucans or β-D-glucans-proteins complex showing greater immunopotentiation activity than free glucans (Wasser, 2010; Zong *et al.,* 2012).

In addition to the well-known antitumor (1-3) – β D glucan, a wide range of biologically active glucans with other structures are described. These glucans are linear or branched molecules with a backbone composed of α-or β-linked glucose units and some of them contain side chains that are attached at different positions (Zhang *et al.,* 2007*;* Synytsya *et al.,* 2009).

Compared with the intensive investigations of β-D-glucans, the biological activity of α-(1 - 3)-D-glucan, which is especially apparent after chemical and structural modification (carboxymethylation, sulfation, aminopropylation, or hydroxyethylation), has been reported only rarely in the literature. But carboxymethylated fungal α-(1 - 3)-D-glucans have a biological activity potential, i.e. they express cytotoxic or mitochondria metabolism-modulating in *Lentinula edodes*, *Pleurotus ostreatus*, *Piptoporus betulinus*, and *Laetiporus sulphureus* has been recently denoted (Wasser, 2010).

For example, 43 terpenoids were separated from *Ganoderma lucidum*, including 6 new compounds. The chemical structures were elucidated and all of the compounds were assayed for their cytotoxicity against human HeLa cervical cancer cell lines. The C-3 carbonyl group, the double bonds ($\Delta^{7,8}$, $\Delta^{9,11}$), the type of the side-chain and the number of hydroxyl group all played an important role in the structure–activity relationships (Cheng *et al.,* 2010).

Besides a typical structure-function relationship that can be found between the anti-tumor activities and structural characteristics of β-D-glucans, a wide range of polysaccharides with different molecular masses ranging from $1x10^4$ to $1x10^6$ kDa can be isolated during extraction. This molecular weight difference can cause diverse immunomodulatory effects and it is, generally, found that those polysaccharides with higher molecular weight ($1x10^5$ kDa or above) would exert stronger anti-tumor and immunomodulatory effects (Wong *et al.,* 2011).

1.2.3 Anti-tumor activity and cellular mechanism

The importance of polysaccharides in tumor treatment was first recognized more than 100 years ago when it was found that certain polysaccharides could induce complete remission in patients with cancer. Since antitumor activity of macrofungal polysaccharides was first published by Chihara in the 1960's, researchers have isolated structural diversified polysaccharides with strong antitumor activity. Unlike traditional antitumor drugs, they produce antitumor effect by activating various immune responses in the host causing no harm to the body (Zhang *et al.,* 2007).

In time mushroom polysaccharides have already shown widely inhibitory effects towards many kinds of tumors suchs as sarcoma 180 solid cancers, Ehrlich solid cancer, sarcoma 37, Yoshida sarcoma and Lewis loung carcinoma (Zhang *et al.,* 2007).

The proposed mechanism by which mushroom polysaccharides exert antitumor effect include: 1) prevention of the oncogenesis by oral administration of polysaccharides isolated from medicinal mushrooms (**cancer-preventing activity**); 2) enhancement of immunity against the bearing tumors (**immuno-enhancing activity**); and 3) direct antitumor activity to induce the apoptosis of tumor cells (**direct tumor inhibition activity**) (Zhang *et al.,* 2007).

Figure 6 shows the protein-bound polysaccharide K (PSK) isolated from *Coriolus versicolor,* a major studied polysaccharide and the different ways it is known to act (Maehara *et al.,* 2012). First, PSK improves host immunocompetence by inhibiting of or neutralizing immunosupressive substances that are increaed in cancer. Second, it activates immune cells such as lymphocytes, either directly or by regulating the production of various cytokines, and third, acts directy in cancer cells.

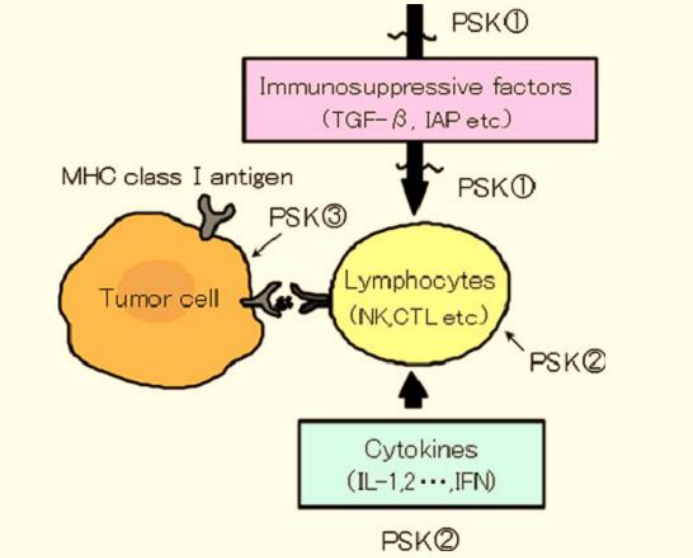


Figure 6 - Tumor microenvironment and actions of PSK. (1) Suppressed production or neutralization of immunosuppressive factors. (2) Activation of immune cells and regulation of cytokine production. (3) ´Direct action on tumor cells [induction of apoptosis, enhanced expression of major histocompatibility complex (MHC) class I antigen] (Maehara *et al.*, 2012)

The **cancer preventing activity** of medicinal mushroom polysaccharides owes its mechanisms to the immunopotentiation of polysaccharides. This cancer preventing activity has been observed in farmers whose main occupation was producing medicinal mushrooms such as *Agaricus blazei* in Brazil. These farmers cancer death rate was 40% lower than in the general population. Consequently, an animal study was conducted by comparison of the administration of an ordinary diet (control) and a *A. blazei* diet (test). After the inoculation with tumors, the number of mice that developed cancer on the test diet was significantly lower than the ones in the control group, indicting the cancer preventing activity of polysaccharides (Zhang *et al.*, 2007).

Mushroom polysaccharides exert their antitumor action mainly via activation of the immune response of the host organism **(immuno-enhancing activity**). They help the host adapt to several biological stresses and exert a nonspecific action supporting some or all of the major systems (Zhang *et al.*, 2007). They enhance a host mediator immunomodulatory response activating the immune system to fight against cancer (Wong *et al.*, 2011). Mushroom polysaccharides have been shown to produce over 50% reduction in tumor size and prolong the survival time of tumor bearing mice (Zhang *et al.*, 2007).

Direct tumor inhibition activity, has also been documented in many mushroom polysaccharides. Even though the anti-proliferative effect of polysaccharides towards tumor lines *in vitro* remains unclear, some studies indicate that incubation of polysaccharides with tumor cells could alter the inner cell signaling expression. Cell cycle arrest and induction of apoptosis could explain the *in vitro* anti-proliferative effect of polysaccharides. These results suggest that mushroom polysaccharides not only stimulate the proliferation of T lymphocytes and the immune function through the immunopotentiation but also have a direct action on the tumor cells even though little is known about its direct effect on cancer cells (Zhang *et al.*, 2007).

Recent studies on direct cytotoxicity have indicated that polysaccharides can directly inhibit the cancer cell proliferation in a dose- and time-dependent manner mediated through processes like up-regulation of p21 and of a pro-apoptosis Bax protein and down-regulation of cyclin D1 (Zhang *et al.*, 2007).

Cancer cells originate from normal cells and a novel agent that can select and destroy only the transformed cells without affecting the normal ones will be an ideal chemotherapeutic agent against cancer. In this respect, the mushroom polysaccharides and polysaccharide – protein complexes should be ideal because they are non-toxic and their anti-cancer effects are mainly host mediated (Sarangi *et al.*, 2006).

1.2.4 Extraction and purification

Mushroom polysaccharides exist mainly as a structural component of the fungal cell wall, composed of two major types of polysaccharides: a rigid fibrillar of chitin (or cellulose) and a matrix-like β-glucan, α-glucan and glycoproteins. Schizophyllan (glucan chitin complex) a water soluble 1-6 branched β-(1-3) - glucan loosely attached to the outer layer of the cell wall, is secreted to the extracellular matrix where exo- or extracellular polysaccharides (EPS) can be found. These water-soluble glucans fill the outer layer resisting external pressure (Zhang *et al.*, 2007).

In general, the extraction method involves elimination of low molecular weight substances from mushroom material. Selection of an extraction method depends on the cell wall structure, and hot water has been the most popular approach yielding water soluble polysaccharides. Methods can vary based on the structure and water solubility

of polysaccharides, but the basic rule is to break the cell wall from outer layer to inner layer with mild-to-strong extraction conditions (pH and temperature) (Zhang *et al.*, 2007).

Extracted polysaccharides can be further purified using a combination of techniques such as ethanol precipitation, fractional and acidic precipitation with acetic acid, ion-exchange chromatography, gel filtration and affinity chromatography. The ethanol precipitation excludes the impurities from the polysaccharides (Zhang *et al.*, 2007).

The polysaccharide with the broad polydispersity can be fractioned by the methods referred above, yielding polysaccharides with different molecular weights and low polydispersity. It should be noted that particular fractionation procedures scheme in each case depends on the composition of the polysaccharide of the original material involving molecular weight branching degree and pattern (Zhang *et al.*, 2007).

1.2.5 Antioxidant activity

Free radicals play an important positive physiological role but may, at the same time, exert toxic effects (Kozarski *et al.*, 2011). It is well known that reactive oxygen species (ROS) such as hydroxyl radical, superoxide anion and hydrogen peroxide are related to the pathogenesis of various diseases (Cheng *et al.*, 2010).

ROS are mostly generated by normal metabolic processes or from exogenesis factors and agents (Hu *et al.*, 2010), as a side product of oxidative phosphorylation. They participate in the regulation of signal transduction and gene expression, activation of receptors and as nuclear transcription factors, also playing a vital role in phagocytosis (Kozarski *et al.*, 2011).

On the other hand, there is increasing evidence that free radicals may play a causative role in a variety of diseases including heart disease, liver damage, cancer, Parkinson's and Alzheimer's diseases, impairment of immune function, cataracts and muscular degeneration in elderly people (Hu *et al.*, 2010; Kozarski *et al.*, 2011).

It is pertinent to note that antioxidant defense systems may only partially prevent oxidative damage. Hence, there is currently a great interest in using dietary supplements

containing antioxidants with a new view to protect the components of the human body from oxidative damage (Jayakumar *et al.,* 2011).

The antioxidant status of the human body reflects the dynamic balance between the antioxidant defense and prooxidant conditions (Kozarski *et al.,* 2011). The oxidative damage to DNA, proteins and other cellular determinants seem inevitable when the concentration of ROS exceeds tolerability of cells (Sun *et al.,* 2004) and impaired physiological functioning may occur, resulting in diseases and accelerated ageing (Kozarski *et al.,* 2012). Anti-oxidants could attenuate this oxidative damage of a tissue indirectly by increasing the cells' natural defenses and/or directly by savaging the free radical species (Sun *et al.,* 2004).

Many synthetic chemicals such as phenolic compounds are found to be strong radical scavengers, but they usually have side effects. For this reason, natural antioxidants are generally preferred in food applications (Kozarski *et al.,* 2012).

Natural antioxidants occur in fruits, vegetables, mushrooms and other natural edible resources. In general, several categories of antioxidants, including phenolic compounds, vitamins, proteins, peptides and amino acids, are widely used as ingredients in dietary supplements and have been investigated for the prevention of diseases such as cancer, coronary heart disease and even altitude sickness (Kozarski *et al.,* 2012).

Among sources of various natural antioxidants, mushrooms have a prominent role. Extracts prepared from mycelium and fruiting bodies of non-toxic (i.e. edible) *Agaricus bisporus, Lentinula edodes, Ganoderma lucidum, Pleurotus sp.* among other mushroom species, show antioxidative activity *in vitro* and may have potential for application *in vivo* (Kozarski *et al.,* 2011; Kozarski *et al.,* 2012).

Polysaccharides are potentially useful biologically active ingredients for pharmaceutical use, such as immune regulation, anti-radiation, anti-blood coagulation, anti-cancer, anti-HIV and hypoglycemic activities. Mushroom derived polysaccharides like lentinan, schizophyllan and krestine have been accepted as immunoceuticals in Japan, Korea and China (Kozarski *et al.,* 2011).

Antioxidant supplements or foods containing high concentrations of antioxidants may help to reduce oxidative damage. Identification of new antioxidants remains a highly active research area (Kozarski *et al.,* 2011).

Among the large resource of fungi, medicinal higher Basidiomycetes, are unlimited sources of biologically active polysaccharides as well as fruit bodies and cultured mycelia. Again, the most bioactive mushroom polysaccharides are β-(1-3) (1-6) glucans (Kozarski *et al.,* 2011), although a wide range of biologically active glucans of other structures have been described (Kozarski *et al.,* 2012).

The hydroxyl radical (OH•) is the most reactive of the ROS and as such can induce severe damage to adjacent biomolecules. The superoxide radical ($O_2^{\bullet -}$), which is generated in numerous biological reactions, is also a highly toxic specie. Although $O_2^{\bullet -}$ radicals cannot directly initiate lipid oxidation, they serve as potential precursors of highly ROS, such as the OH• (Jayakumar *et al.,* 2011).

In the present work, we decided to investigate the anti-oxidative activity of intrapolysaccharides (IPS) of our extracts by assaying the superoxide radical scavenging.

As we have seen, it is extremely advantageous and valuable to explore novel anti-tumor products from medicinal mushrooms (Liang *et al.,* 2011).

1.3 Olive leaves

In recent years, traditional Arab-Islamic herbal medicine has been gaining interest in the scientific community, and more specifically, regarding cancer treatment (Zaid *et al.,* 2012)

The olive tree (*Olea europaea*) is an evergreen tree, from the Oleaceae family, (Zaid *et al.,* 2012) with elongated leaves, present in the Mediterranean region for over 7000 years ago (Lalas *et al.,* 2011).

Throughout the history, *Olea europaea* leaves have been heavily exploited for the prevention or the treatment of hypertension, carcinogenesis, diabetes, atherosclerosis and several other traditional therapeutic uses (Bouallagui *et al.,* 2011; Zaid *et al.,* 2012). Historically, olive leaf has also been used as a remedy for fever and other diseases such as malaria. (Lee *et al.,* 2009)

The incidence of cancer overall in the Mediterranean basin has been thought to be the lowest compared to the other parts of the world. This is mostly accounted for the incidence of cancer of the breast, endometrium, prostate, colon and leukemia. (Bouallagui *et al*,. 2011; Zaid *et al.,* 2012)

Figure 7 - *Verdeal* olive tree leaves
(http://www.quintadoromeu.com/index.php?p=paginas&op=azeite&idioma=eng)

Almost all of the 16 countries bordering the Mediterranean Sea are sharing a common Mediterranean dietary pattern characterized by a high consumption of fruits and vegetables, bread, wheat and other cereals, olive oil and fish. These food supplies are highly rich in bioactive com pounds such as vitamins and polyphenols. A large body of epidemiological evidence, together with data from animal and *in vitro* studies, has brought the proof of polyphenols cancer-preventive properties (Bouallagui *et al.*, 2011).

Typical phenols of the *O. europaea* are the secoiridoids, complex molecules with a molecular weight up to 600 composed of one or two hydroxiaromatics linked to several other non aromatic compunds (Leonardis *et al.*, 2008).

The major active components in olive leaf are known to be oleuropein and its derivatives such as hydroxytyrosol and tyrosol, as well as caffeic acid, p-coumaric acid, vanillic acid, vanillin, luteolin, diosmetin, rutin, luteolin-7-glucoside, apigenin-7-glucoside, and diosmetin-7-glucoside (Lee *et al.*, 2009; Han *et al.*, 2009) .Concentration

of phenols is several times higher in leaves when compared to several oil fractions, and its content varies with time and climate. (Han *et al.,* 2009)

The lower incidence of certain cancers is most likely associated with the antioxidant activity of active ingredients of the olive oil. Oxidative stress has been shown to contribute to cancer development, and antioxidants are believed to reduce the risk of mutagenesis and carcinogenesis. Hydroxytyrosol (HT) or 3,4 – dihydrophenylethanol, is a very bioactive alchoolic ortho-diphenol, component of the secoroiroids. Several studies have demoonstrated its antioxidant and antimicrobial activities, which bennefict in general the cardio-vascular system and prevent several human diseases (Leonardis *et al.,* 2008) throughout its capacity to protect cells from hydrogen peroxide damage and DNA peroxy nitrite-induced damage by blocking cell cycle progression at the G1 phase and inducing apoptosis (Zaid *et al.,* 2012)

Native HT can only be found free in nature with the exception of ripened olive leaves, where the hidrolization of its main component, the oleuropein, occurs (Leonardis *et al.,* 2008). It is found in leaves and fruits as the most important constituent of oleuropein (Han *et al.,* 2009).

In vivo and *in vitro* studies on the activity of oleuropein have found that, in addition to antioxidant properties, it has antiangiogenic action and inhibits cell growth, motility, and invasiveness, for example in lung cancer (Zaid *et al.,* 2012).

Oleuropein was also found to cause cell rounding, which disrupts the cell actin cytoskeleton, providing direct antitumor effects due to cell disruption. In i*n vivo* animal studies, rapid tumor regression was observed hen mice were given one percent oleuropein in drinking water. Saturated animal fats and polyunsaturated plant fats in the diet have been implicated in colon, breast, prostate, and several cancers. The vast usage of olive oil in the mediterranean diet may explain its aparent cancer-protective effect, rather than the amount of fat consumed. Furthermore, in recent study, evaluating the antioxidant and antiproliferative activity of water and methanol olive leaves extracts in cancer and endothelial cells, olive leaf crude extracts were found to inhibit proliferation of cell from a human breast adenocarcinoma, cells from human urinary bladder carcinoma (T-24), and cells from bovine brain capillary endothelial tumor (Lee *et al.,*2009).

2 Thesis aims

In the present study, we analyzed polysaccharide extracts from fruiting bodies and mycelia of several mushroom strings such as *Ganoderma*, *Pleurotus, Irpex, Bjerkandera*, *Piptoporus,* and their biological activity, i.e. free radical scavenging activity, and cytotoxic effect of their polysaccharide content against four tumor cell lines. Phenol compounds from different olive leafs were also analyzed and its effect on human carcinoma cells verified.

It was our intent to perform a systemic study globing some of the following procedures:

1) *In vitro* growth of human carcinoma cell lines, as well as obtaining parameters such as cell viability.
2) Extraction of intra and extra polysaccharides from several mushroom strings such as *Ganoderma, Pleurotus, Irpex, Bjerkandera*, *Piptoporus,* and dosage of protein and polysaccharide content, trough coomassie blue and phenol-sulfuric acid methods.
3) Addition of different concentrations of such polysaccharide extracts to the carcinoma cell lines to study cell viability in their presence through the tetrazolium dye (MTT) colorimetric assay.
4) Phenol extraction of lyophilized and fresh green olive leaves, from 5 different Portuguese varieties of *Olea europaea – Madural, Cobrançosa, Arbequina, Verdial* and *Galega*.
5) Addition of different concentrations of such phenolic compounds to the carcinoma cell lines to study the cell viability in their presence through the tetrazolium dye (MTT) colorimetric assay.

3 Materials and Methods

3.1 Sampling

From this moment on and until the end of this dissertation, fungi samples will be represented as following: *Ganoderma apllanatum* – Ga; *Ganoderma carnosum* – Gc; *Pleurotus ostreatus* – Po; *Pleurotus eriinges* – Pe; *Irpex lacteus* –Il; *Bjerkandera adjusta* – Ba; *Piptoporus betulius* – Pb.

The name of the fungus is often followed by (**α**) in which **α** symbolizes the biomass origin, (1) and (2) for mycelia growth in medium 1 and 2 respectively, (m) from when the biomass was removed from the fruiting body of full grown mushrooms, (s) fungus grown in straw and (r) grown in rice husk (both (s) and (r) were already grown in the straw and rice husks when delivered to analysis).

Figure 8 - *Pleurotus ostreatus* grown mushroom sample

As far as olive samples are concerned, the five varieties are represented by name, *Madural; Cobrançosa; Arbequina; Verdeal and Galega* followed by (a) in younger leaves and (b) in older leaves.

3.2 Mushroom cultivation for production of polysaccharides

Mushroom mycelia cultivation in laboratory was elaborated following some of the rules presented by Gern *et al.,* 2008.

As far as laboratory mushroom mycelia growing strains, a piece of agar plated fungi (i.e *Basidiomycetes* strain) was transferred to an Erlenmeyer flask containing one of the two medium types described in Table 1.

The inoculation growth was accomplished by the transfer of agar plated fungi to a 500 mL Erlenmeyer containing 100 mL of culture medium. The culture was incubated and left to grow at 25ºC and 150 rpm for a week. The culture content was then transferred to a 2L Erlenmeyer containing 500 mL of the similar culture medium. The culture was left to grow another week under the same experimental conditions of encubation.

Glucose is used as a carbon source, and nitrogen source varies from medium 1 – yeast extract, to medium 2 – tomato pomace.

Table 1 - Growth media used in incubation growth of *Basidiomycetes* strain *species.*

Medium 1	Boiled wheat water with 40 gL^{-1} of glucose and 5 gL^{-1} of yeast extract pH 6
Medium 2	Boiled wheat water with 40 gL^{-1} of glucose and 5gL^{-1} of tomato pomace – pH 6

3.3 Isolation of extracellular (EPS) and intracellular (IPS) polysaccharides

Isolation of polysaccharides was accomplished as described by Cui *et al.*, 2007, with some modifications.

Mycelial biomass from the culture broth was separated by centrifugation at 5.000 rpm for an hour, in 250 mL centrifuge flasks.

The culture supernatant was treated with four volumes of 95% (v/v) ethanol to promote precipitation of the EPS. This mixture was left overnight at 4ºC. The EPS were recovered by centrifugation at 20.000 rpm for 45 min, and the pellet was re-suspended in a minimal volume of 50 mM phosphate buffer pH 7.0 (2ml for EPS and 1 ml for IPS).

The mycelial biomass pellet was re-suspended in one volume of distillated water (w/v) and the mixture was heated in a water bath (100ºC) for 6h to release the IPS. The IPS in the extract was recovered using the same procedure as the EPS recovery.

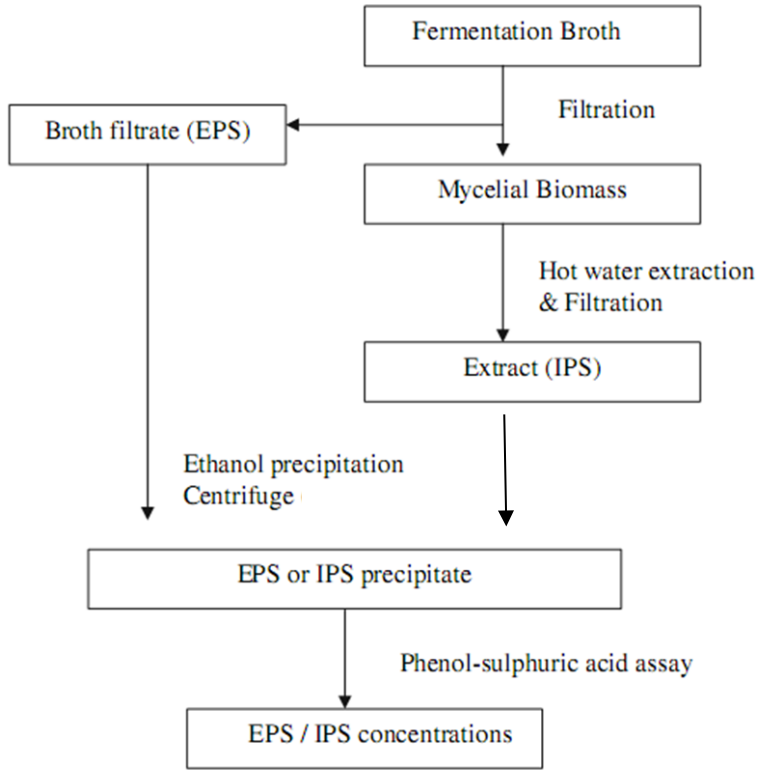


Figure 9 - Isolation of extracellular and intracellular polysaccharides (adapted from Cui *et al.*, 2007)

EPS and IPS levels were quantified by the phenol/sulphuric acid method using glucose and poligalacturonic acid as standards. The key steps involved in EPS/IPS isolation and quantification are shown in Figure 9.

3.4 Polysaccharide determination

Among many colorimetric methods for carbohydrate determination, the phenol–sulfuric acid method is the easiest and most reliable method for measuring neutral sugars in oligosaccharides, proteoglycans, glycoproteins, and glycolipids since phenol in the presence of sulfuric acid can micro determinate sugars and their methyl derivatives, oligosaccharides, and polysaccharides. The phenol-sulfuric acid method is widely used because of its sensitivity and simplicity (Dubois *et al.,* 1956; Masuko *et al.*, 2005

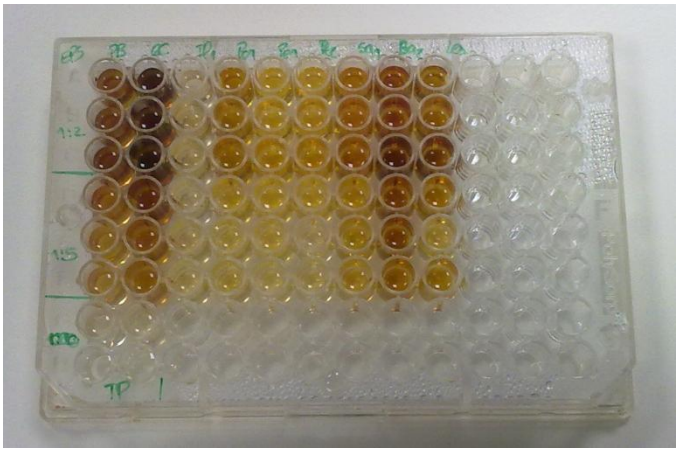


Figure 10 - Plate representing the Phenol - Sulphuric acid method

The quantification of polysaccharides was accomplished using the method described by Masuko *et al.*, 2005. This method was developed in order to reduce the amounts of chemicals used and is performed in a microtiter plate format, (Figure 10) which allowed to increase the number of samples to be assayed such as the column chromatographic fractions.

In a well of a 96-well flat-bottomed polysorp microtiter plates, 150 µl of concentrated sulfuric acid were rapidly added to 50 µl of sample to cause maximum mixing, followed immediately by the addition of 30 µl of 5% phenol in water. After incubating for 5 min at 90º C in a static water bath , the microtiter plate was cooled to room temperature for 5 min in another water bath and wiped dry to measure the absorbance at 490 nm by an microplate reader BIO-RAD model 680. All assays were carried out in triplicate.

Standard curves were carried out by using anhydrous D - glucose and polygalacturonic acid as standards.

3.5 Protein Assay

Quantification of protein was carried out by protein-dye binding method described for the first time by Bradford, 1976, and then by Sedmak and Grosseberg in 1977.

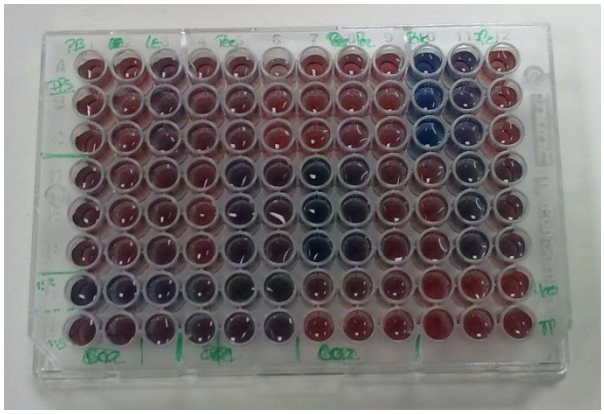


Figure 11 - Coomassie G250 brilliant blue assay

This method, represented in Figure 11, is based on the principle that, under certain conditions, acidic and alkali groups of proteins react with organic dyes forming colored precipitate, and so coomassie brilliant blue G 250 in dilute acid, which possesses a brownish-orange color reacts with protein amino groups, converting into an intense blue color.

In order to measure the protein content, polystyrene 96 well plates were used. The reaction occurred by mixing 100µl of coomassie brilliant blue G250 in 0.06% HCl 0.6 N to 100 µl of the appropriate sample. This mixture was then incubated at room temperature for about 5 minutes before reading its absorbance at 655 nm in a microplate reader BIO-RAD model 680.

The standard curve was carried out by using bovine serum albumin (BSA) solution as standard.

3.6 FTIR analysis

Fourier Transformed Infrared spectroscopy (FTIR) is used to investigate the vibrations of molecules and polar bounds between the different atoms. Structures of polysaccharides, such as monosaccharide types, glycosidic bonds and functional groups, can be analyzed using the FTIR spectroscopy (Yang & Zhang, 2009).

Infrared radiation (IR) refers broadly to that part of the electromagnetic spectrum between the visible and microwave regions. Of greatest practical use to the organic chemist is the limited portion between 4000 and 400 cm^{-1} (Silverstein *et al.*, 2005).

Although the IR spectrum is characteristic of the entire molecule, it is true that certain groups of atoms give rise to bands at or near the same frequency regardless the structure of the rest of the molecule (Silverstein *et al.*, 2005).

An FTIR analysis of intrapolysaccharides from *Ganoderma carnosum* (s) and *Pleurotus eryngii* (m) as well as extrapolysaccharides from *Ganoderma apllanatum* (1) and *Pleurotus ostreatus* (1) was carried out in a Bruker Vertex 70 in KBr pellets with a resolution of 2 cm^{-1} in a range of 500–4000 cm^{-1}.

3.7 Purification of polysaccharides by gel filtration chromatography

Extrapolysaccharides from *Ganoderma apllanatum* (1) and *Pleurotus ostreatus* (1) were purified by gel filtration chromatography on a Sephacryl S-300 HR column (1 cm × 100 cm), previously equilibrated and eluted with 50 mmol L^{-1} phosphate buffer (pH 7.0) at a flow rate of 30 mL h^{-1}.

Fractions (3ml) were collected and analyzed for polysaccharide and protein contents.

After collection each fraction was submitted to the phenol-sulphuric acid, the coomassie protein assay and a measure of absorbance at 200 and 280 nm to detect the presence of polysaccharides and proteins respectively.

3.8 Purification of Polysaccharides by affinity chromatography

Affinity chromatography was performed in 96 well microtiter plate as shown in Figure 12.

Figure 12 - Affinity chromatography columns in 96 well microtiter plates

250 µl of epoxy-activated Sepharose 6B-urea, embedded in 0.001% (m/v) sodium azide, were added to each well. About 15 min were given to the resin to compact as much as possible. A quick centrifuge for 2 minutes at 2500 rpm was performed to consolidate.

Supernatant was recovered and discarded. 150 µl of 50 mM phosphate buffer pH 7.0 was added to each well, centrifuged for 2 min at 2500 rpm and supernatant discarded four times as part as the equilibrium phase

Addition of 100 µl of polysaccharide extract (diluted in buffer solution) to each column (well) was performed and allowed to "sink in" for 15 minutes, with at least 3 refluxes performed.

In the washing process, the plate was then again centrifuged 2 min at 2500 rpm and now 100µl of the supernatant were removed, but collected in the next row of wells (supernatant from well A1 is deposited in well B1).

The washing process is repeated 3 times saving the supernatant in all following rows (as continuing the example, in C1, D1 and E1).

At the fourth time the washing 50 mM phosphate buffer was substituted by the elution buffer (25 mM phosphate buffer with 2M KCl).

This buffer allowed the molecules grouped with the resin to detach and release into the elution buffer, which we continued to remove from the resin column and transfer to new wells. This procedure was again repeated 3 times.

After that time, a phenol-sulphuric acid assay was performed, on the supernatant recovered in the wells, in order to dosage the release of the polysaccharide throughout the washing and elution phase.

3.9 Superoxide radical scavenging

The $O2^{\bullet -}$ is considered to be a highly potent oxidant, and can react with all biomolecules operative in liver cells. The $O2^{\bullet -}$ are produced *in vivo* and can result in the formation of H_2O_2 by the dismutation reaction.

In vitro $O2^{\bullet -}$ were generated from dissolved oxygen through the phenazine methosulfate (PMS) - b-nicotinamide adenine dinucleotide (NADH) – nitro blue tetrazolium (NBT) system.

PMS/NADH coupling reduces NBT, which increases absorption at 560 nm. (Liu *et al.*, 1997; Kodali & Sen, 2008)

In order to optimize this method 25 Mm Tris-HCL buffer, pH 8.0, was added to 780 mM NADH and to 500 µM NBT in a proportion of 4:1:1. 60 µl of this mixture was then added to 20 µl of polysaccharide extract sample in a 96 wells microplate with an automatic multichannel micropitete.

To start the reaction, 10 µl of PMS 100 µM, were added to the well and the reaction mixture was incubated at room temperature for 5 min. Absorbance was measured at 550 nm in a microplate reader BIO-RAD model 680, and Ascorbic Acid (AA) was used as positive control.

The scavenging activity (%) was calculated from the following equation:

$$[1-(\mathbf{A}_{sample} / \mathbf{A}_{blank})]*100$$

were $\mathbf{A}_{blank}$ is the absorbance of the control reaction and $\mathbf{A}_{sample}$ is the absorbance of the sample reaction.

In order to investigate if the time affected the results, kinetic studies were performed between 5 and 30 minutes.

As described by Sun *et al.*, 2004 and Kodali and Sen, 2008, ascorbic acid was used in a concentration of 200 µg/mL and revealed an $O2^{\bullet -}$ scavenge of about 80%.

All assays were carried out in triplicate.

3.10 Extraction of phenolic compounds

The extraction of phenolic compounds was performed according to Han *et al*, 2009. Lyophilized and fresh green olive leaves, provided by Professor Eduardo Rosa, from the department of Agronomy, UTAD were collected from 5 different Portuguese varieties of *Olea europaea – Madural, Cobrançosa, Arbequina, Verdeal* and *Galega* – and two types of leaves were extracted – Older and young - in order to find out if ageing would affect their biological properties.

10 g of fresh or lyophilized leaves were extracted in 100 ml of 70% ethanol for 2 weeks in the dark. Extracted solution [10% (w/v)] was than filtered with a Millipore, (pore size 0.22 µm) filter unit.

3.11 Phenolic compounds Assay

Experiments were performed according to Cicco *et al.*, 2009, with some modifications. 100 µl of properly diluted samples, calibration solutions or blank were pipetted into separate eppendorfs tubes and 100 µl of Folin - Ciocalteau reagent was added to each tube. The mixture was mixed well and allowed to equilibrate.

After exactly 2 min, 800 µl of a 5% (w/v) sodium carbonate solution was added. The mixture was swirled and put in a static water bath at 40 °C for 20 min. Then, eppendorfs tubes were rapidly cooled on the rocks and the colour generated was read at its maximum absorption, i.e.765 nm in the case of gallic acid (standard solution). The

absorbance was measured in a glass cuvette in a Thermo Evolution 300LC UV/visible spectrophotometer.

All assays were carried out in triplicate.

3.12 Human carcinoma cell lines

Cell lines were acquired from European Collection of Cell Cultures (ECACC)

Table 2 - Cell lines characteristics (data obtained by ATCC and Sigma) (http://www.lgcstandards-atcc.org/ and http://www.sigmaaldrich.com)

Cell Type	Specie	Tissue	Organ	Disease	Morphology
HeLa Ac-Scc	*Homo sapiens*		Cervix	Cervix adenocarcinoma containing human papilloma virus (HPV-18)	Epithelial
A549	*Homo sapiens*		Lung	Lung carcinoma	Epithelial
A431	*Homo sapiens*	Skin	Epidermis	Epidermoid carcinoma	Epithelial
OE21	*Homo sapiens*	Skin	Oesophagus	Squamous carcinoma of mid oesophagus	Epithelial

3.13 *In vitro* anti-tumor activity assay

Cell lines were grown in culture flasks of different volumes, depending on the volume and growth rate of each cell line. Cells were maintained in the appropriate culture medium in a CO_2 incubator at 37º C containing 5% CO_2.

For cell lines A432, A549 and HeLa AC-SCC, cells were grown in DMEM (Dulbeccois Modified Medium) containing gentamycin and10% fetal bovine serum (FBS) whereas the cell line OE21 was grown in RPMI (Roswell Park Memorial Institute medium) medium, supplemented by FBS, penicillin and streptomycin.

In order to remove the cells from the flask and distribute them in 96 well plates, we first need to remove the supernatant of medium, since the cells in flask grow in an

epithelial adherent manner (forming a carpet). Previously warmed phosphate buffered saline (PBS) at 37 ºC, was used to wash the cells. Trypsin was then added, to release cells from the flask carpet, and the flask was mixed and incubated for about 2 – 3 minutes again at 37ºC. This procedure was repeated as many times as necessary to completely remove the cells from the flask.

Pre-warmed medium was then added to the flask to remove cells from the flask and transferred to a centrifuge tube. Since trypsin is toxic to the cells, we add a minimum amount FBS to the medium removed with cell content, in order to inactivate the toxic properties of trypsin during the next steps.

Cell containing medium was centrifuged for 6 minutes at 2000 rpm, the supernatant was removed and fresh medium was added to the pellet which was re-suspended. Subsequently, cell concentration was determined in these samples.

Cells were treated with 0.1 (w/v) trypan blue in PBS and viable cell concentration was determined by the Neubauer improved chamber method.

3.14 Cell viability method

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenly tetrazolium bromide (MTT) colorimetric assay, an established method of determining viable cell number in proliferation and cytotoxicity studies.

This assay is based on the cleavage of the yellow salt, MTT, to form a soluble blue formazan product mostly by mitochondrial enzymes, and the amount of formazan produced is directly proportional to the number of living, not dead cells, present during MTT exposure. Since the MTT assay is rapid, convenient, and economical, it has become a very popular technique for quantification of viable cells in tissue culture. (Sylvester, 2011)

Cells were seeded on 96-well tissue culture plates at a density of 5.5×10^4 cells/well and allowed to attach for 24 h. Polysaccharide extracts, (IPS, EPS) were added to the cultures at different concentrations. The PBS used to dissolve the extracts also served as a control. Phenolic compounds where dissolved in culture medium. Cultures were grown in a tissue culture incubator at 37 ◦C containing 5% CO_2 for two days.

All polysaccharides and phenolic compounds samples were filtered by a Millipore 0.45 µm filter before addition to the cells, in order to maintain sterility.

Cell growth was determined by MTT assay. 20µl of MTT (5mg/mL) were added to the wells and left incubating at 37◦C and 5% CO_2 for 4 hours, before removing all the content and the MTT product was solubilized in 200µl of dimethyl sulfoxide (DMSO).

The survival rate of human carcinoma cell lines was assayed by measuring the optical density in a microplate reader at 550 nm microplate reader BIO-RAD model 680 (corrected by subtraction with the reference absorbance at 650 nm)

Results were expressed as the inhibition ratio (ϕ) of human carcinoma cell line proliferation as follows:

$$\phi(\%) = [(A - B)/A] \times 100$$

where A and B are the average numbers of viable tumor cells of the control (i.e. medium) and test samples respectively. All assays were carried out in triplicate. (Silva *et al.*, 2012)

A 3 gL^{-1} starch solution was used as a polysaccharide positive control.

3.15 Data analysis

All data acquired was analyzed and treated with Excel. Media, standard deviations and errors were calculated. Sigma Plot was used to draw graphics.

4 Results and Discussion

4.1 Polysaccharide, sugar and protein content

In Table 3 we can find polysaccharide, protein and sugar (glucose) content of all extracts used in the development of this work.

Table 3 - Polysaccharide, sugar and protein content (g L^{-1}) of samples used during the elaboration of the present work.

	IPS			EPS		
	Polysaccharide g L^{-1}	**Sugar g L^{-1}**	**Protein gL^{-1}**	**Polysaccharide g L^{-1}**	**Sugar g L^{-1}**	**Protein gL^{-1}**
Gc (s)	3.2578	1.1533	_____	0.0375	0.0896	_____
Pb (r)	6.7171	2.2960	_____	0.3136	0.1808	_____
Pb (s)	5.4041	1.8623	_____	0.0908	0.1072	_____
Gc (r)	1.4506	0.5564	_____	0.3266	0.1851	_____
Pb (l)	8.33	3.13	14.15	36.38	12.79	70.71
Gc (l)	12.94	4.66	12	45.54	15.81	67.86
Il (1)	2.0664	0.77	9.02	1.8310	_____	95.9
Il (2)	5.96	2.35	17.9	_____	_____	26.08
Po (1)	0.84	0.66	8.89	14.29	5.49	106.49
Pe (1)	2.7383	_____	_____	3.8777	3.60	26.08
Pe (2)	3.6871	4.59	11.9	3.6871	_____	39.09
Ga(1)	20.78	7.25	9.95	11.35	4.52	26.82
Ga (2)	12.5	4.53	4.79	_____	_____	37.87
Ba (2)	42.2	14.35	4.42	42.54	14.76	36.33
Pe (m)	0.46	0.53	24	_____	_____	19.8
Po (m)	0.76	0.63	18.81	_____	_____	58.71
Le (1)	6.16	2.42	35.9	50.81	17.57	26.08

4.2 Superoxide radical scavenging

In Table 4 and Table 5, results of SO scavenging, for IPS extracts, crude and precipitated respectively, are presented in a matter of average scavenging.

Table 4 - SO scavenging (%) of IPS (results are shown as average ± SD, and all experiments were carried out in triplicate); AA (200 µg mL^{-1}) was used as a positive control.

	Il (1)	Po (1)	Pe (2)	Po (m)
No dilution	56.83 ± 0.006	50,07 ± .000	81.13 ± 0.007	22.08 ± 0.006
1:2	39.25 ± 0.011	36.54 ± .007	68.78 ± 0.006	17.7 ± 0.027
1:4	15.39 ± 0.025	31.73 ± 0.025	39.26 ± 0.012	_____
1:16	4.28 ± 0.009	8.58 ± 0.022	11.42 ± 0.007	10.65 ± 0.016
1:64	_____	_____	11.68 ± 0.006	9.17 ± 0.005
	Pe (m)	**Ga (1)**	**Ga (2)**	**Il (2)**
No dilution	63.42 ± 0.005	67.22 ± 0.002	76.57 ± 0.000	50.50 ± 0.009
1:2	51.30 ± 0.002	45.71 ± .006	50.66 ± 0.003	27.72 ± 0.007
1:4	25.37 ± 0.014	23.87 ± .005	20.46 ±0.012	14.85 ± 0.006
1:16	1.13 ± 0.003	4.79 ± 0.012	4.62 ±0.005	5.06 ± 0.004
1:64	4.64 ± 0.014	_____	_____	1.65 ± 0.006
	Pb (s)	**Pb (r)**	**Gc (r)**	**Ba (2)**
No dilution	70.78 ± 0.003	68.83 ± 0.004	73.43± 0.004	76.57 ± .001
1:2	54.70 ± 0.006	59.21 ± 0.003	55.39 ± 0.009	63.53 ± 0.006
1:4	24.80 ± 0.008	27.23 ± 0.017	30.13 ± 0.013	21.78 ± 0.007
1:16	12.23 ± 0.020	48.78 ± 0.001	10.08 ± 0.003	6.60 ± 0.010
1:64	4.19 ± 0.007	16.45 ± 0.026	5.45 ± 0.033	3.47 ± 0.019

Acid ascorbic as a positive control showed 87.95 ± 0.002 SO scavenging

In both cases, as visible in both tables 4 and 5, IPS extracts scavenge $O2^{\bullet -}$ in a concentration dependent manner, as reported by Sun *et al.*, 2004.

We can also see that IPS extracts that were obtained by ethanol precipation (Table 5) can, in some cases, show less scavenging activity than the ones that did not. This has leads us to believe that, although the polyssacharide concentration of the sample is

higher (due to the precipitation), in the first case, we are dealing with crude extract, and are not aware of all the mixture components, therefore non- polyssacharide components may also increase the SO scavenging. In the cases were the samples precipitaded have higher scavenging values we atribute such cause to the increased concentration of relevant polyssacharides in the sample.

Table 5 - SO scavenging (%) of IPS undergone ethanol precipitation; (results are shown as average ± SD, and all experiments were carried out in triplicate); AA (200 µg mL^{-1}) was used as a positive control.

	Ba (2)	Il (1)	Il (2)	Pe (2)
No dilution	61.96 ± 0.008	48.55 ± 0.011	80.86 ± 0.001	48.24 ± 0.009
1:2	42.05 ± 0.018	37.48 ± 0.004	39.22 ± 0.013	37.32 ± 0.002
1:4	24.52 ± 0.007	28.81 ± 0.003	38.45 ± 0.000	18.60 ± 0.001
1:16	23.93 ± 0.011	12.33 ± 0.004	23.71 ± 0.002	8.97 ± 0.020
1:64	3.42 ± 0.029	11.82 ± 0.001	8.71 ± 0.007	1.43 ± 0.010
	Ga (1)	**Ga (2)**	**Po (1)**	
No dilution	80.99 ± 0.002	65.92 ± 0.005	50.07 ± 0.000	
1:2	34.91 ± 0.013	50.72 ± 0.005	36.54 ± 0.007	
1:4	32.89 ± 0.002	30.24 ± 0.011	31.73 ± 0.025	
1:16	22.50 ± 0.005	20.69 ± 0.008	8.58 ± 0.022	
1:64	8.79 ± 0.039	6.33 ± 0.003	_____	

Acid ascorbic as a positive control showed 85.89 ± 0.002 SO scavenging

In order to investigate the effect of time on the reaction, measurements of absorbance were carried out every 5 minutes, for 30 minutes.

In Figure 13, we show two cases that exemplify what happens to the SO scavenging of our samples during the 30 minutes length. The data allows us to see, that when dealing with samples with higher concentration, time does not influence greatly the results but, when in turn, we analyze lower concentrations of the sample (greater dilutions) the variation of SO scavenging (%) ranges from 16% to 0%, in some cases.

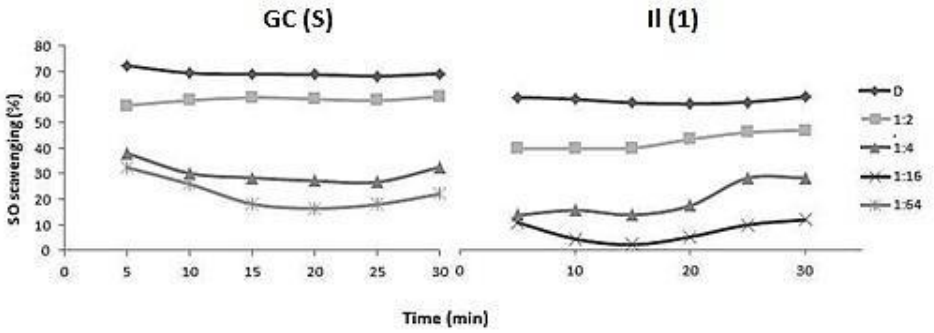


Figure 13 - SO scavenging time dependence tests in *Ganoderma carnosum* (s) and *Irpex lacteus* (1); legend shows dilutions used, (D strands for direct, no dilution was applied).

During this experiment, we concluded that the optimum values of SO Scavenging can be found between 15 and 25 minutes, after the reaction, depending on the polysaccharide extract in question.

4.3 Purification of polysaccharides by gel filtration chromatography

Figure 14 and Figure 15 show the chromatograms obtained for *Ganoderma apllanatum* (1) and *Pleurotus Ostreatus* (1) after purification by gel filtration.

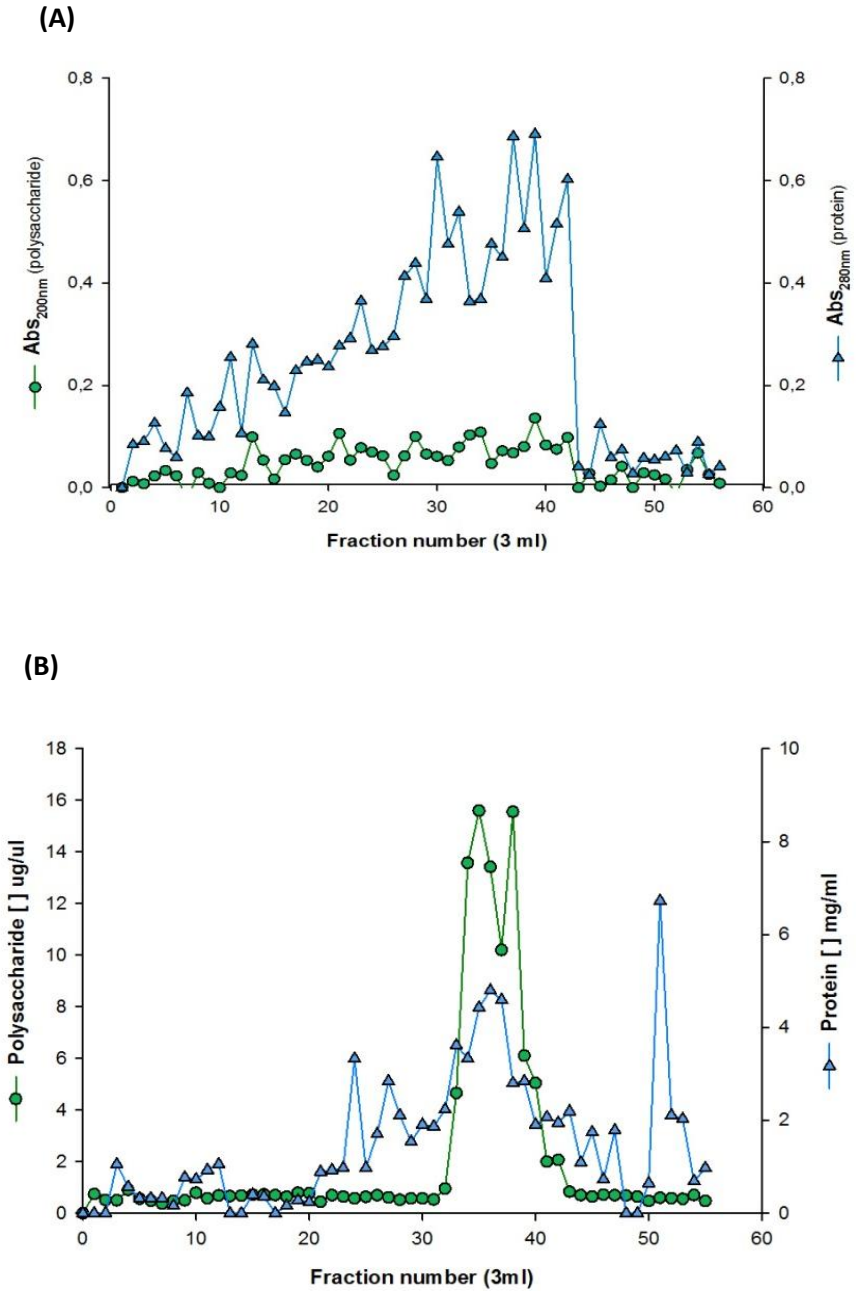


Figure 14 - Chromatographic behavior of *Ganoderma apllanatum* (EPS) on Sephacryl S-300 HR column (A) absorbance at 200 nm and 280 nm; (B) protein and polysaccharide measurements

(A) of both figures shows the polysaccharide and protein results obtained by absorbance measure at 200 nm and 280 nm, wile (B) shows the polysaccharide and protein content determined by the phenolic – sulphuric and coomassie G250 brilliant blue method.

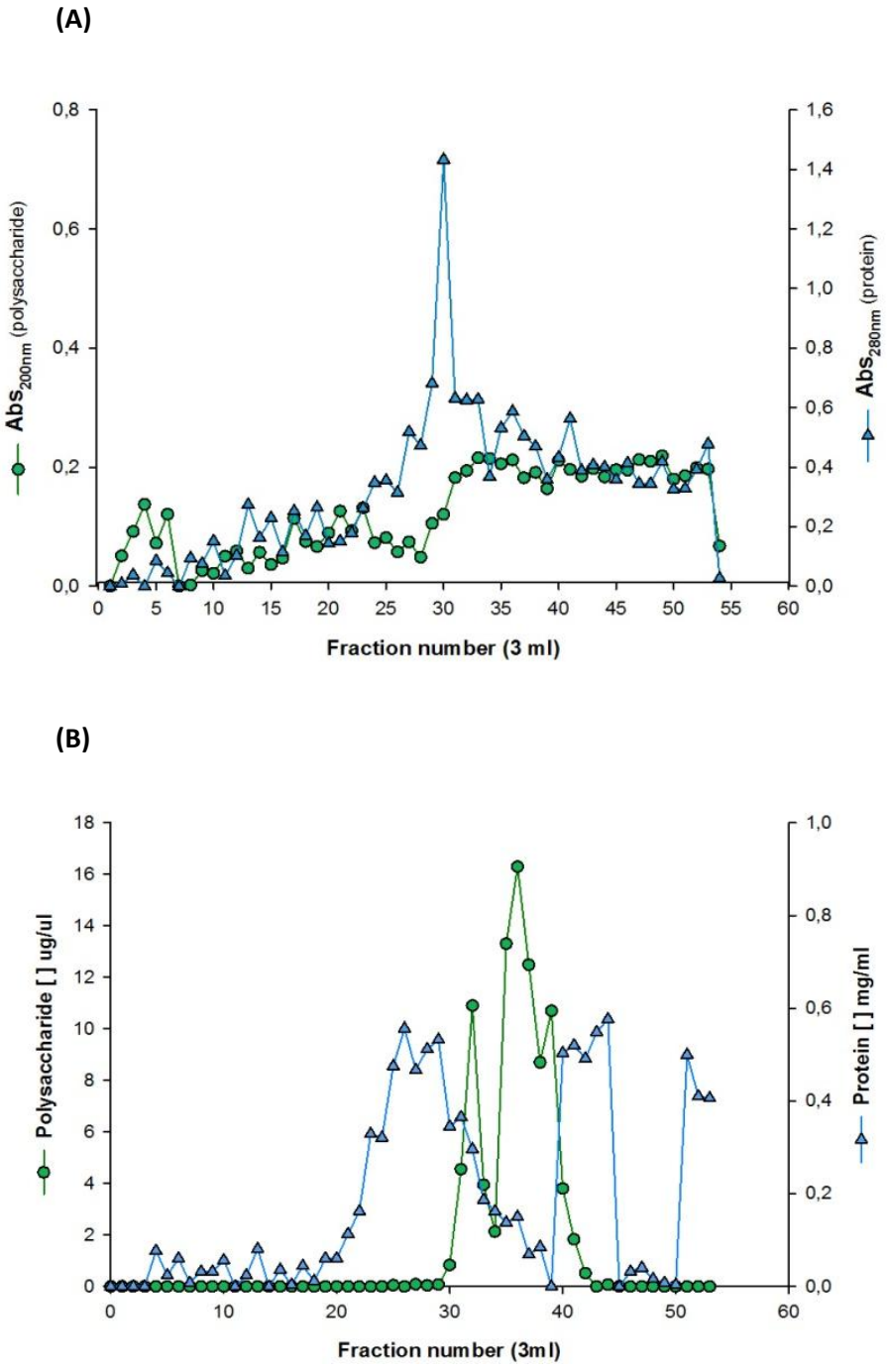


Figure 15 - Chromatographic behavior of *Pleurotus ostreatus* (EPS) on Sephacryl S-300 HR column; (A) absorbance at 200 nm and 280 nm; (B) protein and polysaccharide measurements

We can see that the fractions between 30 and 40 in both purifications were the fractions with the highest content in polysaccharide, and were thus the chosen to apply to human cell lines.

4.4 Purification of polysaccharides by affinity chromatography

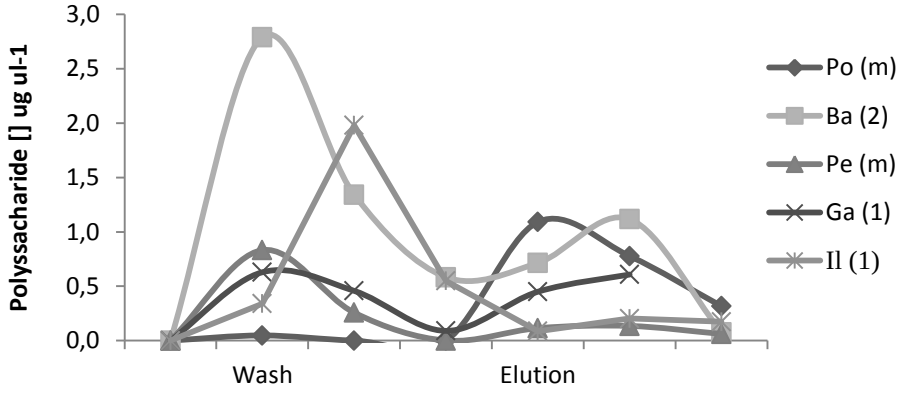


Figure 16 - Affinity chromatography on epoxy-activated Sepharose 6B-urea of polysaccharides from several *Basidiomycetes* strains in urea activated resin with phosphate buffer pH 7.0.

As showed in

Figure **16**, a few of our samples responded positively to urea activated resin.

In Po (m) we were able to recover more than 90% of the sample, and all of it in the elution phase, which suggests that this sample exhibited total adsorption to the resin.

Ba (2) showed a 70% recovery with a distribution of 70% - 30% (wash – elution). These data suggest that this sample exhibited partial adsorption to this chromatographic matrix; there is decrease in the amount recovered in the wash and an uprising in the beginning of the elution phase.

In Pe (m) and Ga (1) we were only able to recover about 50% of the total sample. In the first case we had a 70% - 30% recovery and in the second case a 50% - 50% recovery in the washing and elution phases, respectively. Both are considered cases of partial adsorption of polysaccharides to this chromatographic matrix.

We are also able to see other samples that did not adsorb, such as Il (1). In this case the recovery was 100% but almost everything was recovered in the washing phase.

This type of activated urea was used in other works in samples like soy beans. Since we knew little of the total composition of our mixture, we were able to find out that between the urea activated resin and the polysaccharides present there was a total or partial adhesion to the carbonyl groups available, in some of our samples.

While trying to improve this technique, we used wells of a 96 well microtiter plate as columns.

4.5 FTIR analysis

The IR spectra obtained for our samples are represented in the following Figure 17 and Figure 18.

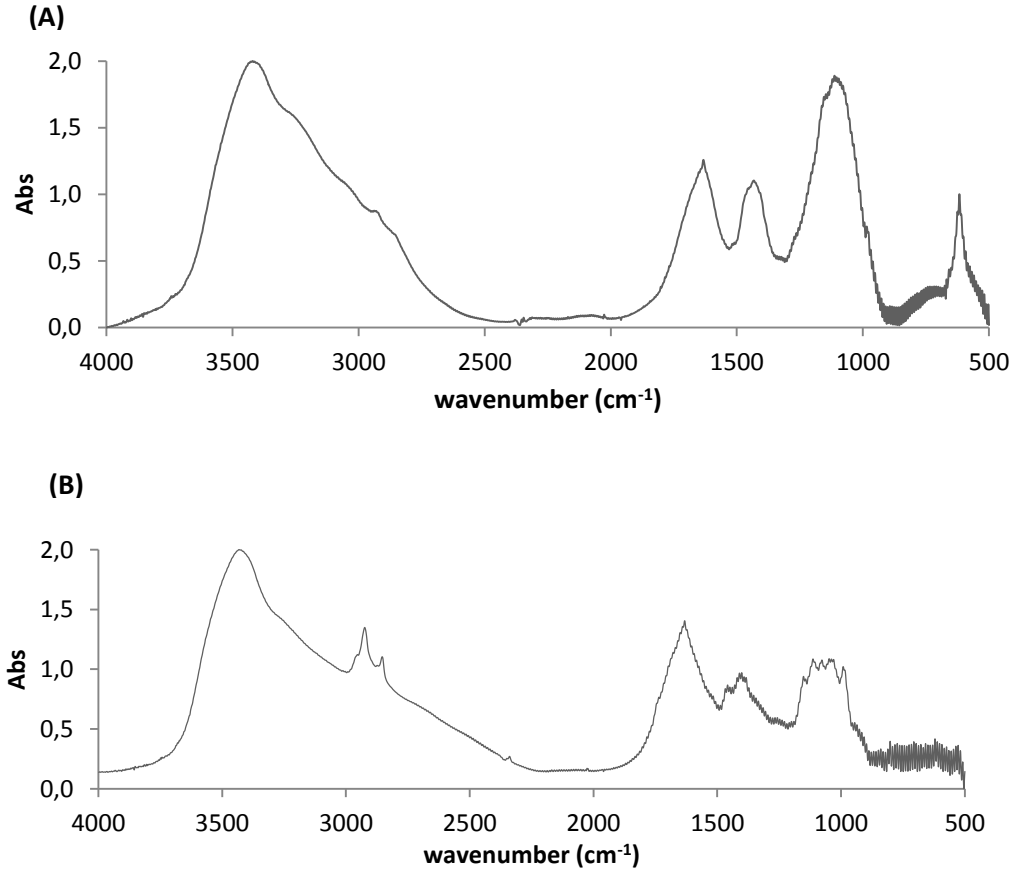


Figure 17 – FTIR Spectra of IPS from (A) *Ganoderma carnosum* (straw) and (B) *Pleurotus eryngii* (mushroom).

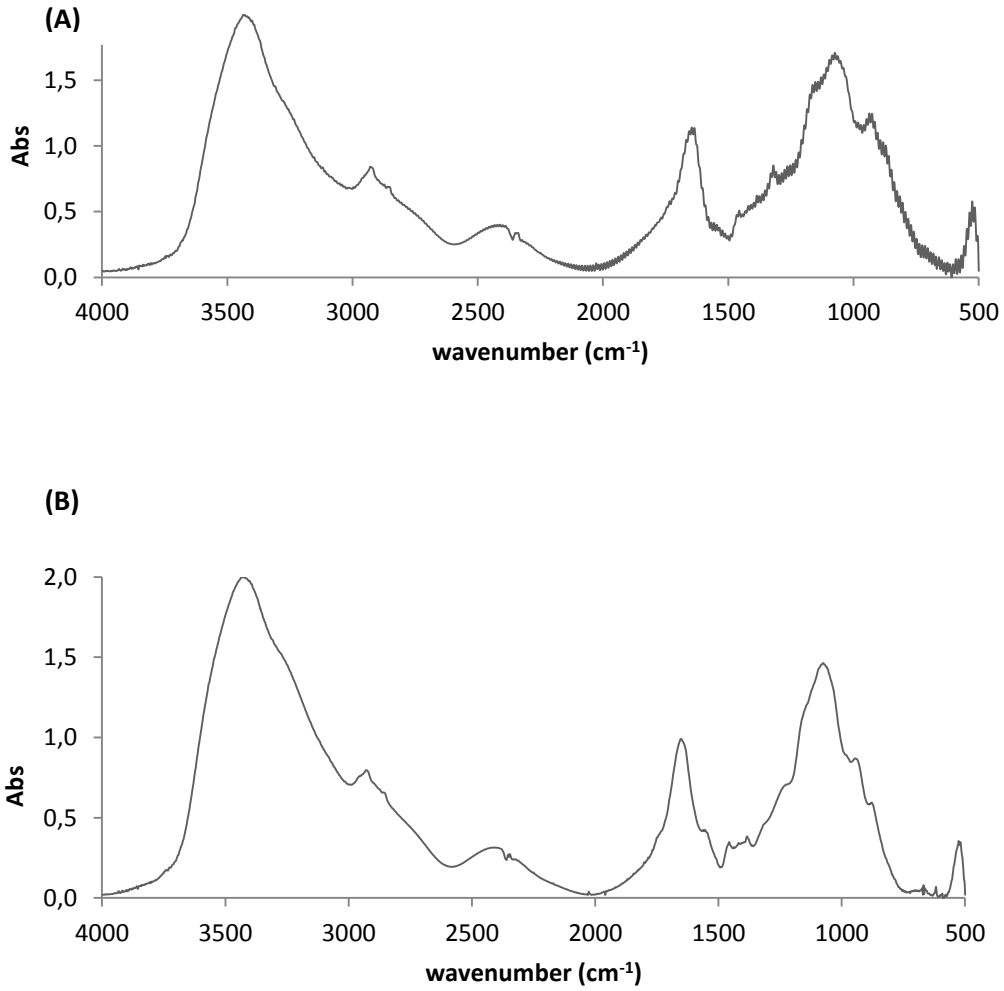


Figure 18 - FTIR spectra of EPS from (A) *Ganoderma apllanatum* and (B) *Pleurotus ostreatus* grown in medium 1.

Intracellular polysaccharides from *Ganoderma carnosum* grown in straw (Figure 17(A)) revealed some characteristic absorption bands. A strong band at 3420 cm^{-1} due to -OH stretching vibration is evident. This band can also be accompanied by the extension of the -OH group belonging to the H_2O molecule. Such broad absorption bands centered near 3300 cm^{-1} are representative of carbohydrates. The band observed at 2973 cm^{-1} is associated to C-H extension. The band at 1632 cm^{-1} corresponds to vibration of the carbonyl group (C=O) of amide I, while the band at 1435 cm^{-1} is due to C-OH in plane bending. The stretching band at 1095 cm^{-1} in the IR spectrum suggests the presence of C-O-C bonds found in glucans. Weak absorption bands 1544 cm^{-1} (amide II) and 1154 cm^{-1} (C-O extension, tertiary alcohol) are also evident. According to Synytsya and co-workers, intense highly overlapped IR bands in the region of 1200 –

950 cm^{-1} (mainly due to C-C and C-O stretching vibrations in pyranoid rings) indicate the presence of polysaccharides as the major component (Radziki and Kalbarczyk, 2010; Synytsya *et al.*, 2009).

The weak C-H extension band at 2973 cm^{-1} can also indicate the presence of amino acid groups.

The IR spectrum of intracellular polysaccharides from *Pleurotus euryngii* (Figure 17(B)) exhibited bands in common with the previous spectrum, namely at 3432 cm^{-1} (-OH stretching); 2853 cm^{-1} (C-H extension); 1151 cm^{-1} (C- O extension, tertiary alcohol) and the overlapped IR bands at 1200 – 950 cm^{-1} (C-C and C-O stretching vibrations in pyranoid rings).

IR spectra of extrapolysaccharides from *Ganoderma apllanatum* (1) (Figure 18 (A)) reveal again a strong band at 3435 cm^{-1} due to -OH stretching vibration. At 2928 cm^{-1} (C-H extension); 2410 cm^{-1} (amino acids); 1645 cm^{-1} (amine I); 1363 cm^{-1} (amine II); 1052 cm^{-1} and 993 cm^{-1} presence of β-glucans and at 515 cm^{-1} presence of α-glucans.

It has been reported by Kozarski *et al.*, 2012, in *Ganoderma apllanatum* spectrum, the presence of a broad band at 3000-3500 cm^{-1} assigned to OH stretching in hydrogen bonds and N-H vibration and a band at 2924 cm^{-1} which is characteristic for CH_2 stretching. The spectrum showed an absorption band at 1690–1750 cm^{-1} that indicated the presence of a free acid or esterified acid C_5O_5 group as the structural component of at least one polysaccharide in the extract.

Figure 18 (B) shows the IR Spectrum of extrapolysaccharides from *Pleurotus ostreatus* (1) with the peaks already described in the other spectra, 3430 cm^{-1} (OH stretching); 2410 cm^{-1} (amino acids); 1652 cm^{-1} (amide I); 1564 cm^{-1} (amide II); 1075 cm^{-1} (β-glucans) and 526 cm^{-1} (α – glucans) (Gutirrrez *et al.*, 1996; Lambert *et al.*, 1998; Synytsya *et al.*, 2009; Wang, *et al.*, 2012).

The weak broad band at 2410 cm^{-1} patent in spectra from Figure 18 may indicate the presence of amino acids in our extracellular polysaccharides extracts.

We can also collect from bibliographic features that, the amide II band, which is due to bending vibrations of N–H groups, is best used for estimation of protein content.

We have seen in state of art that the presence of β –glucans and α – glucans were often associated with the anti-tumor capacity of the polysaccharide. By FT-IR analysis we were able to confirm their presence in our samples.

4.6 Phenolic compounds assay

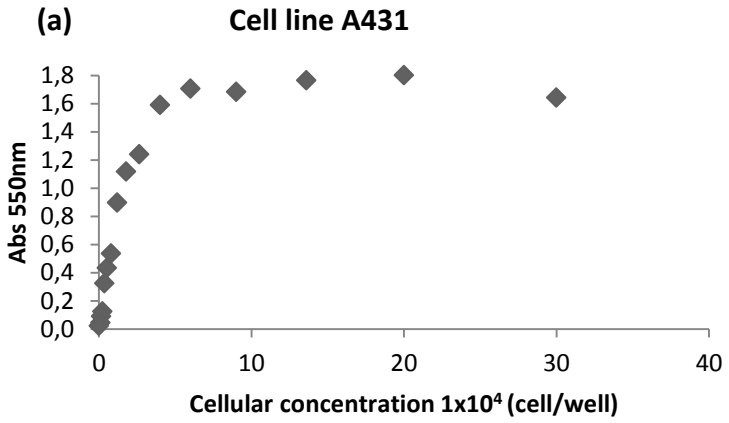
Table 6 – Assay of phenolic compound in fresh leaves and lyophilized samples; as referred (a) and (b) represent younger and older leaves respectively; values are represented as µg/mL.

	Madural		***Verdeal***		***Cobrançosa***		***Arbequina***		***Galega***	
	(a)	**(b)**	**(a)**	**(b)**	**(a)**	**(b)**	**(a)**	**(b)**	**(a)**	**(b)**
Fresh leaves	559	709	321	321	689	364	844	816	257	209
Lyophilized samples	1732	2465	1456	1603	1543	772	1469	1578	1451	1625

The data presented in the Table 6 strongly suggests that lyophilized leaves exhibit higher phenolic contents than fresh leaves. It is also showed, that the proportion however is maintained between young and older leaves in the lyophilized samples when compared to fresh ones.

4.7 *In vitro* anti-tumor activity assay

4.7.1 Standard growth curves for human carcinoma cell lines



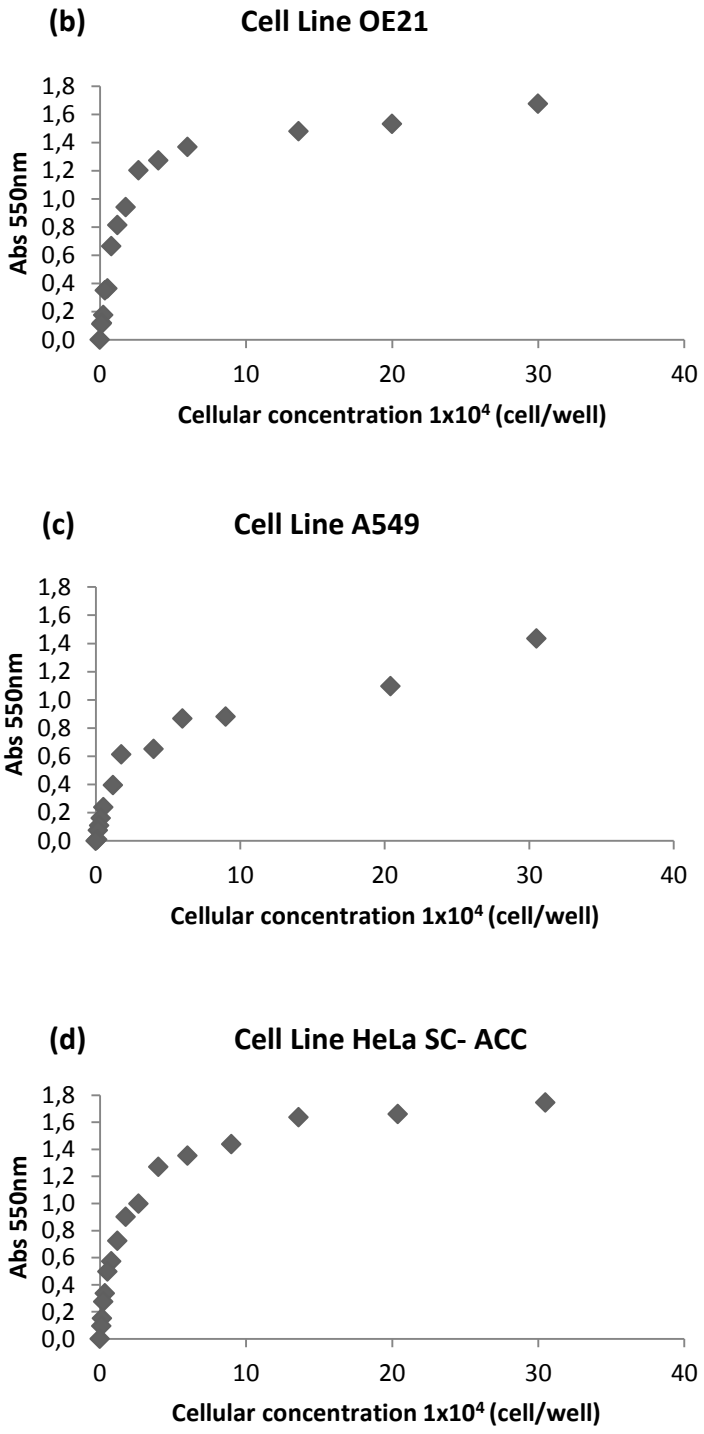


Figure 19 - MTT calibration curves for the 4 human carcinoma cell lines used in this dissertation; values represented are mean of triplicates.

As sugested by Silevester, 2011, viable cell number can be determined most easily in 24 or 96-well culture plates using the MTT colorimetric assay. The number of cells/well is calculated against a standard curve prepared at the start of each experiment, so that the same cell and MTT preparations are used for both the standard curve and the

experiment. Standard curves are prepared by plating various concentrations of isolated cells, previously determined by newbauer improved hemocytometer, in triplicate in the culture microtiter plates. Depending on the type of cell to be counted, standard curves are prepared so that MTT absorbance readings of experimental samples are obtained in the linear part of the curve.

In this way we are able to detemine, if our concentration of $5{,}5x10^4$ cells/well was being correctly determined in all of our four lines, since their behaviour as we can see, is diferent from one to another.

4.7.2 Effect of IPS on cellular proliferation of human carcinoma cell lines

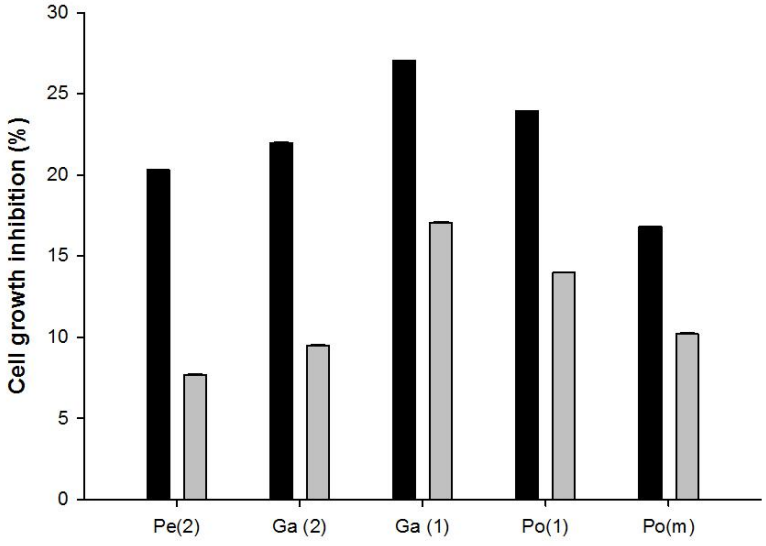


Figure 20 - Cell growth inhibition (%) by addition of IPS 1000ug/mL and 200 ug/mL to the HeLa Ac-Scc cell line; mean of triplicate values and error are represented.

Our values of inhibition are similar to those find in literature, about these types of polysaccharides.

From this point on, we decided to focus on lesser reported data, and decided to investigate the extrapolysaccharides effects.

4.7.3 Effect of EPS on cellular proliferation of human carcinoma cell lines

Cell growth inhibition (%) by addition of EPS to the different cell lines HeLa Ac-Scc, A549, A431 and OE21 and two following dilutions are represented individually in Figure 21, 22, 23 and 24. In each case only the EPS that have showed cell inhibition are represented.

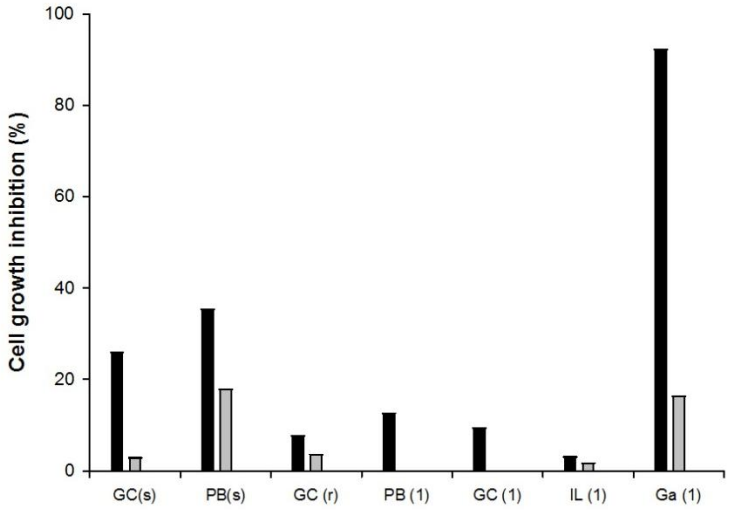


Figure 21 - Cell growth inhibition (%) by addition of EPS to HeLa Ac-Scc; all of the samples represented are, from left to right, undiluted, a dilution of 1:2, and of 1:10; except for Il (1) in which case the best results were obtained with a 1:2, 1:10 and 1:50 PBS dilutions; mean of triplicate values and error are represented.

The data presented revealed that the inhithion of cell growth is dependent on the amount of polysacharide present. As we can see, direct ou higher concentrations may exert inibhitory effect on cell growth, while in higher dilutions that effect may be reduced or totally absent. In all four lines, three dilutions of polysaccharides were represented, an in some cases, only one or two of them exhibited inhibitory effect on human carcinoma cell lines.

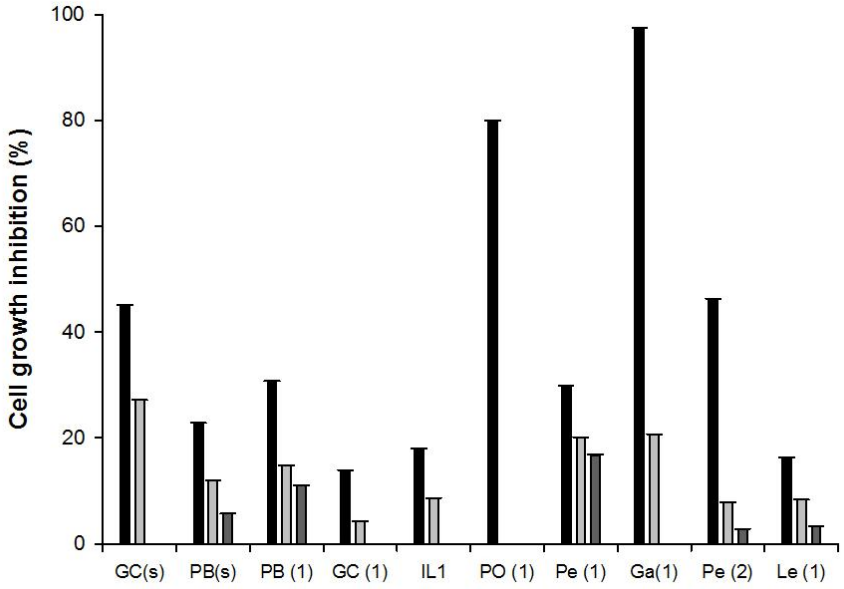


Figure 22 Cell growth inhibition (%) by addition of EPS to A459; Gc (s), Po (1), Pe (2), Pe (1) and Pb (1) inhibitions were obtained from left to right with undiluted, 1:2 and 1:10 dilutions while the remaining Pb (s), Il (1), Ga (1) and Le (1) were obtained also from left to right with a 1:2, 1:10 and 1:50 PBS dilutions.; mean of triplicate values and error are represented

We can also see that, in addition to the amount of polysaccharide, inibithion is also dependent of the cell line type.

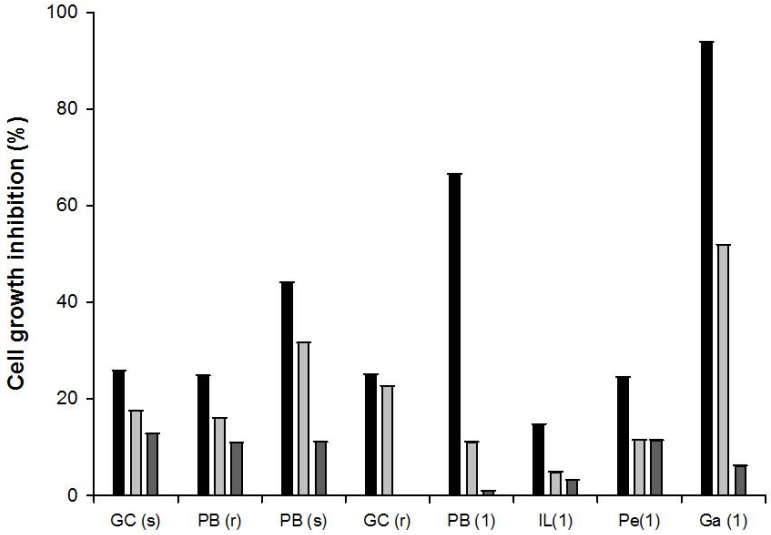


Figure 23 Cell growth inhibition (%) by addition of EPS to A431; from right to left undiluted samples, 1:2 and 1:10 dilutions are represented, except for Gc(s), which is also from right to left a 1:2, 1:10 and 1:50 PBS dilutions.; mean of triplicate values and error are represented

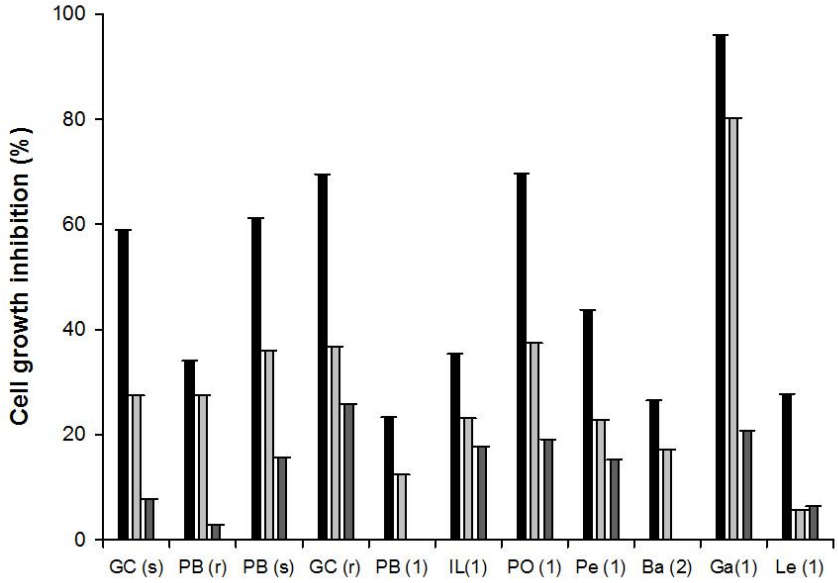


Figure 24 - Cell growth inhibition (%) by addition of EPS to OE21;from right to left direct addition and 1:2 and 1:10 dilutions, with the exception of Le (1) and Pb (1) that were 1:2, 1:10 and 1:50 PBS dilutions.; mean of triplicate values and error are represented.

In some cases a polysaccharide can exert an inibhitory factor in more than one cell line, like Gc (s), Pe (1) or Il (1). A cell line in return can be more sensitive to the effects of a polysaccharide than another. As we can see, almost every EPS tested in the cell line OE21 can exert some kind of inibhitory factor, while in the rest of our cell lines, the response was never that broad.

The data presented above suggest several observations. We can see for exemple that in cell line OE21, Gc (s) only needs a to create the highest inhibition with a dilution of 1:2, while a direct addition in the other three cell lines was not able to produce such an effect, thus corroborating the mentioned fact, that not only does the amount of polysaccharide, but also the cell line characteristics define the inibhitory effect.

We can also conclude that growth medium, as studied by other researchers, like Silva *et al*, 2012, is important not only in the amount of polysaccharide obtained but also in the type of polysaccharide produced, which in the end can tend to exert diferent effects.

As we can see in Figure 24, the polysaccharide Pe, whether it was grown in culture medium one or in culture medium two, can exert inibhitory effect on cell line A549, but with diferent % of inibhition. By using this polysaccharide as an exemple, we can also

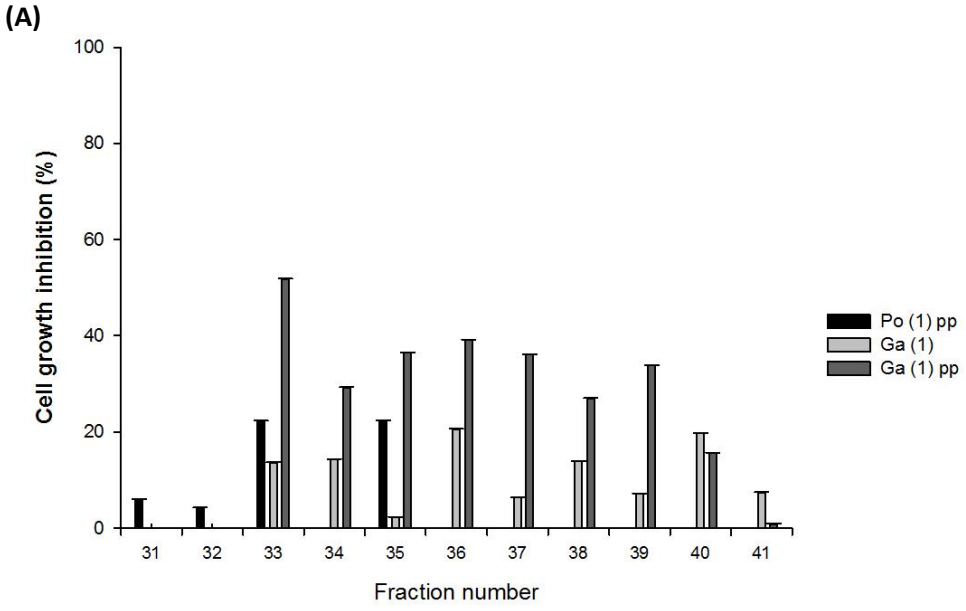
see that the one grown in medium one, can exert inibhitory effect in three of the four lines, while the one grown in medium two was only capable of inihibithing one cell line. The data in Table 3 (page 30) reveales that the sort of polysaccharide formed in the medium is important in it's inibhithory capacity, since the amount of polysaccharide formed is the same in the both media.

The most surprising result in the present work was the powerfull inhibitory effect of EPS Ga (1).This polysaccaride mixture, was able to generate in all four cell lines, a massive inibhitory effect of about 90%. As far as the cell line A549 is concerned, this inhibition was remarkably generated by a 1:2 dilution of this sample.

4.7.4 Addition of cromathographic fractions to the cell lines

After gel purification the chromatographic fractions were added to cell lines A459 and OE21, as shown in Figure 25

For the Po (1) samples we choose the fractions 31 to 42, in the Ga (1) 33 to 42, in correspondence to the higher polysaccharide values shown in data represented in Figure 14 and Figure 15 respectively.



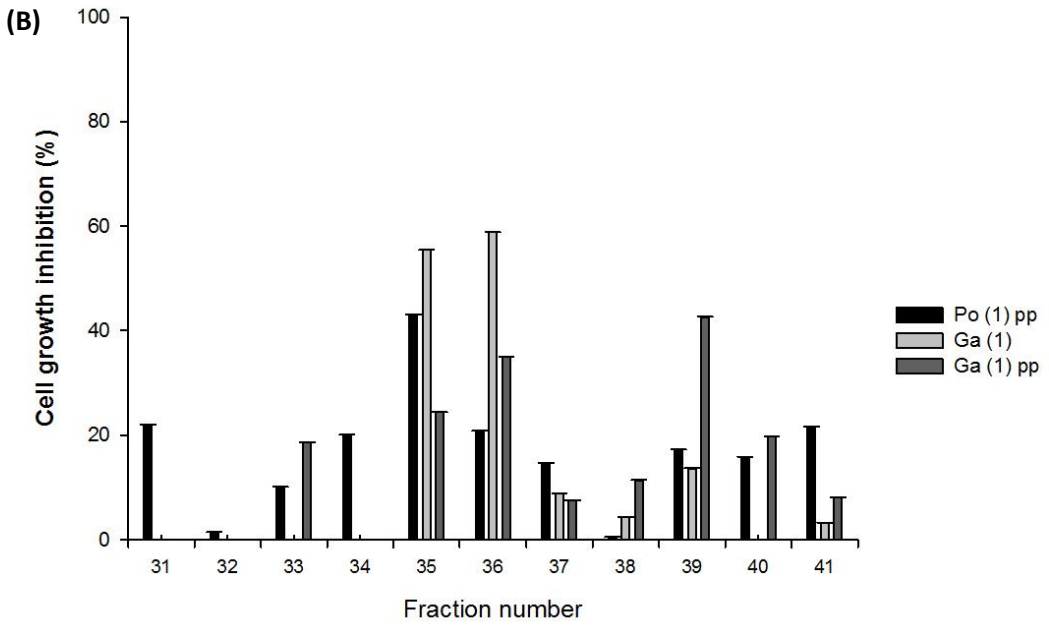


Figure 25 - Application of the gel chromatographic fractions to line **(A)** A549 and **(B)** OE21; mean values of triplicates and error values are represented; fractions described as pp have been purified by alcohol precipitation after the chromatographic extraction.

Although in general none of the fractions achieved the value of inhibition demonstrated by the non-chromatographic samples some came inventively close, almost every time in Ga (1) EPS samples.

This allows us to extrapolate, that some of the components lost, for example, in exclusion volume, although not rich in polysaccharide content, can definitely contribute to its cell anti-proliferative effect.

Posteriorly, our samples were also purified by the 95 % alcohol precipitation described in materials and methods, under EPS recovery. Samples were described as Po (1) pp or Ga (1) pp.

When we analyze the final results, in both cell lines, the chromatographic fractions of Po (1) did not respond as it was expected. In almost every case the inhibition value was very low or inexistent. Po (1) fractions undergone precipitation have however responded better, and were able to cause inhibition, mostly in cell line OE21, a cell line we have already seen, is more sensitive to polysaccharide action.

However, in Ga (1) we were able to obtain inhibition values, and in samples precipitated, that is, more concentrated, the value inhibition is in general higher than in the fractions non undergone precipitation.

Since this has happened to both Po (1) and Ga (1), we assume that yes, concentration of polysaccharide, as it has been extensively reported in literature is important, but the components present, again show an enormous importance. If not let's examine Figure 25 carefully.

The higher polysaccharide content of the Ga (1) fractions added belongs to fractions 34 to 38.

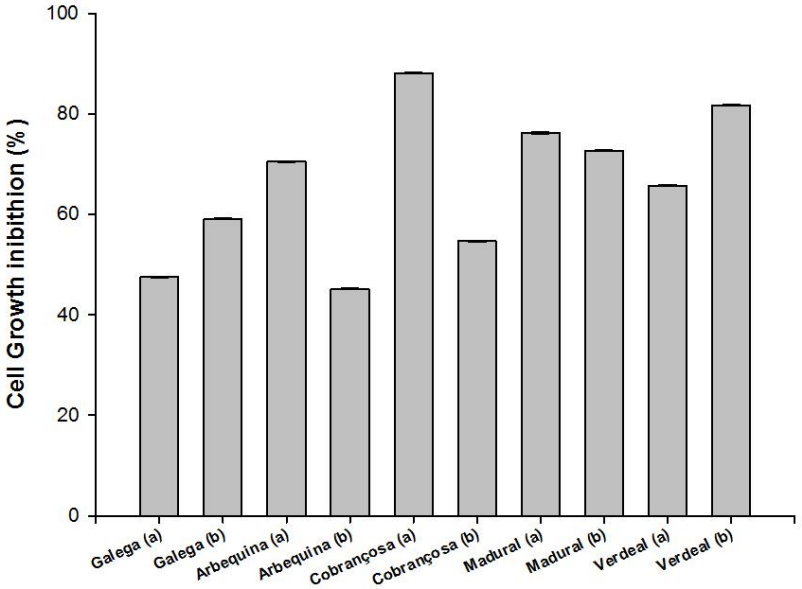
In A549, Figure 25 (A) the values appear much to what we would expect, the higher the concentration, the higher the inhibition rate. But if we analyze the values represented more closely we can see, however, that the higher fractions are not the ones that show the highest inhibition rate. We would expect fractions 35 - 38 - 36 - 34 to show in a decreasing manner, higher levels of inhibition, and fractions 33, 39 and 40 (that have lower polysaccharide content) lower levels of inhibition and that is not what happens. Nevertheless, in this case there are many variables that could have influenced the results as similar as they are.

On the other hand, when analyzing Figure 25 (B) we can further examine the relation between concentration and inhibition, since the fractions with higher polysaccharide content are not the ones that show higher values of inhibition, thus the component eluted at fractions 38 to 40 seems to reveal an important role in cell inhibition, in both cell types.

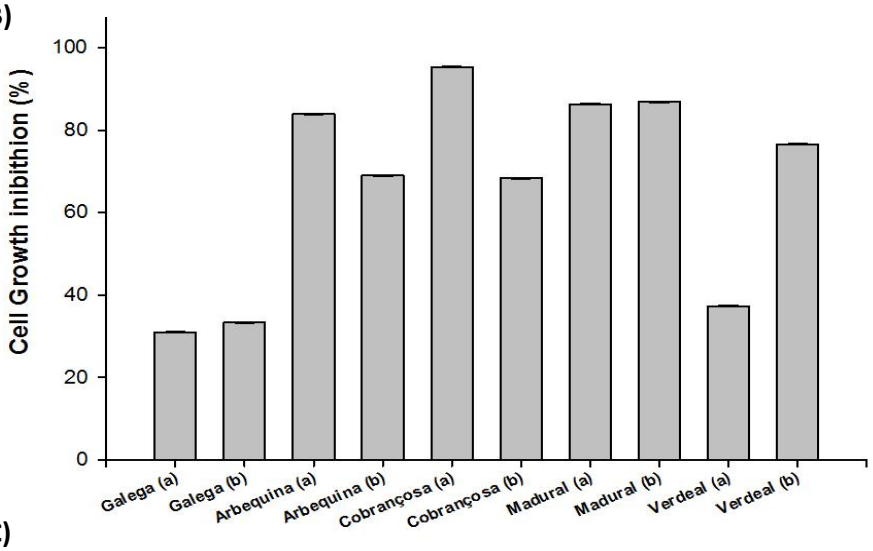
4.7.5 Effect of phenolic compounds on proliferation of human carcinoma cell lines

The effects of phenolic compunds from fresh leaves on the proliferation of the four cell lines are represented on Figure 26 (A), (B), (C) and (D).

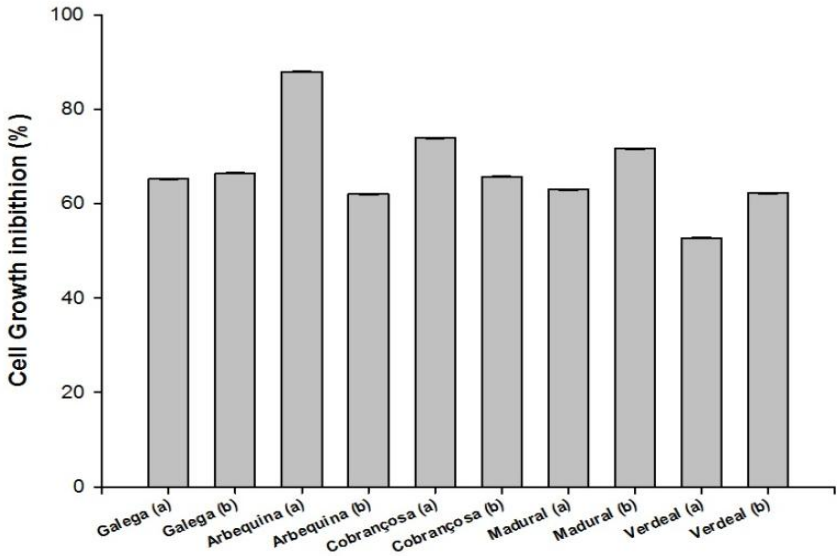
(A)


Cell Growth inibithion (%)
100
80
60
40
20
0
Galega (a)
Galega (b)
Arbequina (a)
Arbequina (b)
Cobrançosa (a)
Cobrançosa (b)
Madural (a)
Madural (b)
Verdeal (a)
Verdeal (b)

(B)


Cell Growth inibithion (%)
100
80
60
40
20
0
Galega (a)
Galega (b)
Arbequina (a)
Arbequina (b)
Cobrançosa (a)
Cobrançosa (b)
Madural (a)
Madural (b)
Verdeal (a)
Verdeal (b)

(C)


Cell Growth inibithion (%)
100
80
60
40
20
0
Galega (a)
Galega (b)
Arbequina (a)
Arbequina (b)
Cobrançosa (a)
Cobrançosa (b)
Madural (a)
Madural (b)
Verdeal (a)
Verdeal (b)

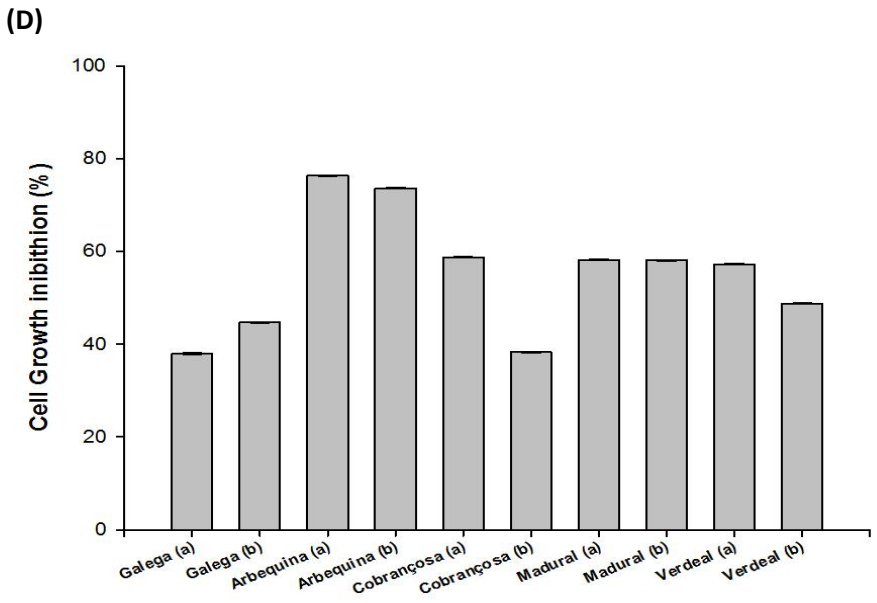


Figure 26 - Cell growth inhibition (%) by addition of olive phenolic compounds extracted from fresh leaves to the different cell lines **(A)** HeLa Ac-Scc; **(B)** A549;**(C)** A431 and **(D)** OE21; young leaves are represented as (a) and older leaves as (b); mean of triplicate values and error are represented;

When confronted with the question are there differences between younger and older leaves, if we reexamine the phenolic content described in Table 6 (page 40), except for the *Cobrançosa* sample in which the phenolic content differs from almost 50% between young and old leaves, the phenolic content of the other species does not differ drastically from one type to the other.

However, the data reported in Figure 26 (A), (B), (C) and (D) have revealed that the inhibition of cell proliferation is not always the same for all cell lines. Again, there are differences and similarities between cell lines.

As far as the *Arbequina* sample is concerned, younger leaves exhibited a higher inhibitory effect than the older ones. On the contrary three out of four times, older *Verdeal* leaves have shown better results. Therefore, the data presented in this work strongly suggest that there are marked differences in both types of leaves which may be due to the presence of different phenolic compounds in such leaves.

The effects of phenolic compunds from liophilized leaves samples on the proliferation of the four cell lines are represented on Figure 27 and Figure 28, and they show a rather interesting pattern.

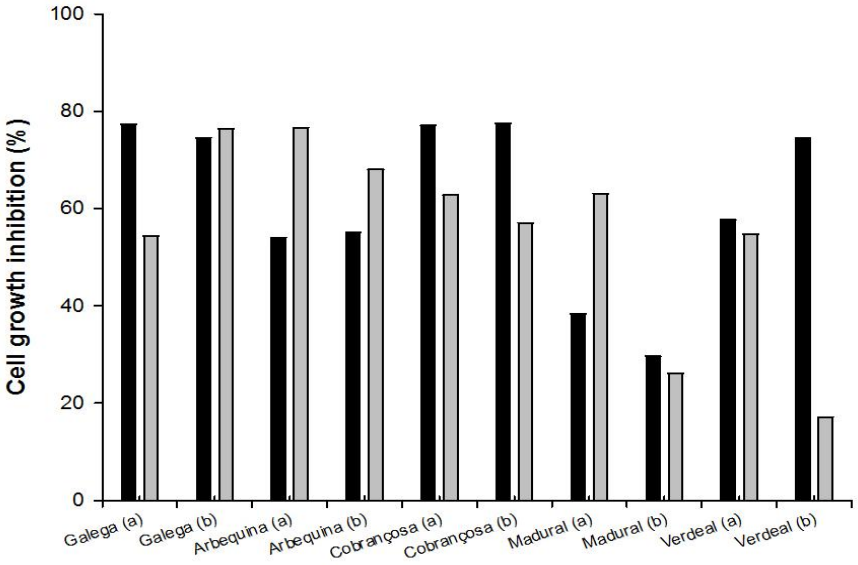


Figure 27 - Addition of lyophilized leaves to A431 cell line. The black bars represent a 1:10 dilution in PBS and gray bars represent 1:50 dilution in DMEM; mean values and error bars are represented.

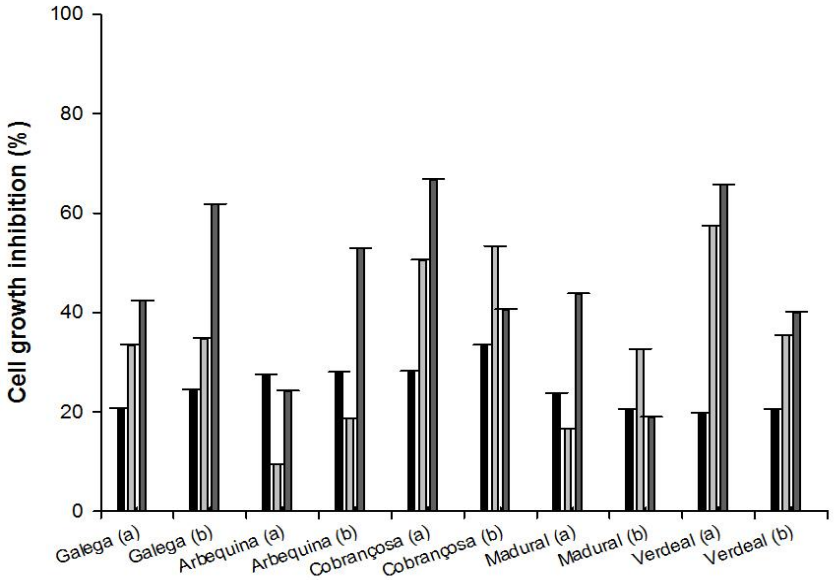


Figure 28 - Addition of lyophilized leaves to A549 cell line. The black bars represent a 1:10 dilution in PBS, light gray a dilution of 1:50 in PBS and dark gray a 1:100 dilution in DMEM; mean values and error bars are represented.

We have seen that lyophilized samples have about 2-3 times the amount of phenolic compounds when compared to fresh olive leaves. When applied in the same manner to the cell lines A431 and A549, lyophilized samples show a distinctive but rather persistent pattern. Up to a certain dilution (in medium) the inhibitory effect seems to be compromised. In almost every case no direct sample (without dilution) showed no inhibition or a rather very low one. When reached a certain dilution however, the inhibition starts to grow.

There were no references in the literature that we could find about this occurrence, but as we have seen in the state of art, several compounds can be found in leaf extract, not only phenols, and thus, it has lead us to believe that we can be in the presence of two antagonist components, and that only when reached a certain point of low concentration of one, can the other yield its place and act as an inhibitor.

Furthermore, in data not shown, if we decrease the cell number per well, this inversion between dilution and inhibition is more accentuated.

5 Conclusions and further perspectives

Cancer, as we have already evidenced, is a resource and life consuming disease that affects millions of people all over the globe. The necessity of complementary therapies with low toxic effects is a goal to be achieved, and the present work had that same objective in view.

Thus we set out to find natural products that can, by means of several characteristics exert that role. As thus, we analyzed several species of mushrooms, and a variety of olive leaves in search of an inhibitory effect on cancer establishment and development.

This work has brought to our attention that mushroom IPSs have great SO scavenging activities and that this factor is time dependent, mainly when dealing with lower polysaccharides concentrations. The anti-oxidative capacity demonstrated by this fact can be one of the major way in which this polysaccharides can act against tumor cells, and help maintain the oxidative equilibrium in our systems.

The major discovery in this project lied in the efficiency of EPS extracts removed from *Ganoderma apllanatum.* In all four cell lines it was capable of exerting an average of 90-95% inhibition of cell growth and thus expansion of tumor cells. This sample deserves to be further investigated by methods that measure more accurately the cytotoxic effects it exerts like cycle cell arrest or apoptosis, cell morphology analysis and it's purification can be obtained by other methods, like HPLC, or mass spectrometry in order to discover as much as possible about the characteristics of this sample, that can be exploited to innovate cancer therapy.

Dealing with olive leaves also showed to be a good bet in further investigation. Our preliminary study report it exerts overall satisfactory inhibitory effect of different intensities depending on the cell line, and most surely the components present in the sample. No considerable differences were found between younger and older leaves, but however, lyophilized samples provided us with a distinctive pattern that deserves further attention. The phenolic compounds should be as polysaccharides analyzed in full, to understand the mechanism behind this odd behavior.

In sum, due to the great amount of samples and the wish to innovate and optimize existing procedures it was not possible to analyze in greater detail the composition of our samples.

More detailing techniques like HPLC (hydrolyze attached), NMR and FTIR are techniques can help us better to understand the composition and conformation of our samples, and in the future, identify and isolate the anti-tumor components.

From our part, we set out to discover if several fungi species and olive leaves phenols cited in literature as having anti-cancer effects on animal tests, were also able to prevent the expansion of four specific types of human carcinoma cells.

The mechanisms by which they act on cell cycle are also extremely variable as we have seen throughout the state of art and trough literature available and thus we recommend enzyme tests, like cycle cell arrest, apoptosis studies, morphology analyses, and Elisa as some of the techniques that can be performed in a near future in continuance of this preliminary study.

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