UNIVERSIDADE FEDERAL DO PARANÁ

SASCHA HABU

ESTUDO DA ATIVIDADE ANTIOXIDANTE E ANTITUMORAL DE BIOCOMPOSTOS DE MACROMICETOS PRODUZIDOS EM FERMENTAÇÃO SUBMERSA EM CO-CULTIVO

CURITIBA

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Tese apresentada como requisito parcial à obtenção do grau de Doutor do Programa de Pós-Graduação em Processos Biotecnológicos. Área de Concentração: Biotecnologia Agroalimentar. Setor de Tecnologia da Universidade Federal do Paraná.

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CURITIBA

2010

AGRADECIMENTOS

APRESENTAÇÃO

Os cogumelos participam da alimentação humana há séculos e o consumo é mais evidente em países orientais. Nos últimos anos, com os avanços nas pesquisas dos cogumelos, cientistas constataram inúmeras propriedades biológicas dos corpos de frutificação. Esse fato contribuiu para difundir o consumo de cogumelos no mundo. E o seu sabor, antes considerado exótico, vem conquistando novos apreciadores e mercados.

No entanto, os cogumelos vão além da gastronomia. Estudos investigam quais metabólitos são bioativos, bem como, testam tecnologias mais rápidas e eficientes para a obtenção dos compostos de interesse.

Atualmente a saúde não visa apenas o tratamento, mas a prevenção de doenças e por isso há uma corrida na descoberta de novas substâncias naturais. Cerca de sete milhões de pessoas morrem de câncer em todo o mundo a cada ano. Segundo as estimativas do Fundo Mundial de Pesquisas sobre Câncer (World Câncer Research Fund International – WCRF), este número deve aumentar para dez milhões em 2020. Com o desenvolvimento de novas tecnologias de fermentação e purificação, os basidiomicetos recebem uma maior atenção como fonte potencial de substâncias bioativas.

A linha de estudo sobre métodos de cultivo e atividades biológicas de cogumelos é desenvolvida há vinte anos no laboratório de Bioprocessos da Universidade Federal do Paraná, sob coordenação do professor Dr. Carlos Ricardo Soccol. Esse documento faz parte da linha pesquisa de propriedades nutracêuticas de cogumelos cultivado em fermentação submersa. Os resultados da pesquisa foram organizados em cinco capítulos com o intuito de informar os dados relevantes do estudo desenvolvido no período de 2006 a 2010.

Os dois capítulos iniciais referem-se às propriedades medicinais de compostos extraídos de cogumelos e sobre fermentação submersa de cogumelos para obtenção de biocompostos. Os capítulos seguintes apresentam resultados referentes às propriedades antioxidantes e antitumorais de compostos bioativos obtidos por fermentação submersa de macromicetos em co-cultivo. As espécies estudadas foram: *Agaricus brasiliensis*, *Cordyceps sinensis*, *Ganoderma lucidum* e *Grifola frondosa*.

FIGURES

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SUMARY

LIST OF SYMBOLS AND ABBREVIATIONS

Ab	*Agaricus brasiliensis*
Cs	*Cordyceps sinensis*
Gl	*Ganoderma lucidum*
Gf	*Grifola frondosa*
ATP	Adenosine triphosphate
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BF	Broth fermented
^{o}C	Degree Celcius
Ca^{+2}	Calcium
CaCl	Calcium Choride
DPPH	2,2-diphenyl-1-picrylhydrazyl
Dw	Dry Weight
EBE	Ethanolic Biomass Extracts
EDTA	Ethylenediamine tetra-acetic acid
EPS	Exopolissacharide
Fe^{+3}	Iron
GAE	Gallic Acid Equivalents
GC	Gas Chromatography
g/L	Grams per Liter
Gal	Galactose
Glc	Glucose
HIV	Human immunodeficiency vírus
HPLC	High-performance liquid chromatography
IL-6	INTERLEUKIN – 6
IFN	INTERFERON
IPS	INTRAPOLISACCHARIDE
kDa	Kilo Daltons
LPB	Bioprocess laboratory
M	Molar
Mg	Miligram

Mg$^+$	Magnesium
Man	Manose
MRSA	Methicillin-resistant Staphylococcus aureus
MTT	(3-(4,5-<u>Dimethylthiazol</u>-2-yl)-2,5-di<u>phenyl</u>tetrazolium bromide
NADH	Nicotine adenine dinucleotide
NK	Natural Killer
Nm	Nanometer
NBT	Nitroblue tetrazolium
NY	New York
O$_2$	Oxygen
OH	Hydroxyl
PBS	Phosphate Buffer Solution
PDA	Potato Dextrose Agar
Ppm	Parts per million
Ph	Potential Hydrogenionic
Rham	Rhamnose
RPM	Revolutions per minute
SD	Standard deviation
TNF	Tumor necrosis factor
UFPR	Federal University of Paraná
USA	United States of America
µg	Micrograms
µm	Micrometer
µM	Micro mol
V/V	Volume/volume
VVM	Air volume/medium
WHO	World Health Organization
Xyl	Xylose

Chapter I

MEDICINAL PROPERTIES OF EDIBLE MUSHROOMS

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Abstract

Mushrooms belong to Fungi Kingdom although in the past, they were classified as plants. The fruiting bodies receive attention for their forms and colors when they are found in nature. The interest in mushrooms is old, Oriental countries use the mushrooms for the treatment and prevention of several diseases such as arthritis, rheumatism, bronchitis, gastritis, cancer, as well as in health and longevity maintenance. Scientists search answers about the action mechanism of mushrooms and about alternative treatments based on natural compounds. Many species are appreciated in culinary around the world because of their good taste and their nutritious potential. There are researches about bioactive compounds such as polysaccharides, proteo-glucans, phenol compounds, nucleotides and their action mechanism to treatment for several diseases. Studies have shown significant results in the immune system improvement, antimicrobial and anti-angiogenic activity and in the treatment of the cancer and high cholesterol. The aim of this chapter is describes any biological properties of mushrooms to the treatment of several diseases.

1 Introduction

Mushrooms belong to a special group of macroscopic fungi. Macromycetes arranged in the phylum Basidiomycota and some of them in the Ascomycota are known as the higher fungi (Moradali *et al.*, 2007, Sicoli *et al.*, 2005). It is estimated the existence of about 140.000 different species of mushrooms in the planet, however, only about 10% is known. Half of them present nutritious properties. 2.000 species of mushrooms are safe and, approximately, 70 are known for presenting some pharmacological properties.

Edible mushrooms are attractive because of their flavor, taste, and delicacy (Diyabalanage, 2008). Although many species of edible mushrooms exist in the nature, less than 20 species are used as food and only 8–10 species are regularly cultivated in significant extent.

Fresh mushrooms can be acquired from grocery stores and markets, including straw mushrooms (*Volvariella volvacea*), oyster mushrooms (*Pleurotus ostreatus*), shiitakes (*Lentinula edodes*) and enokitake (*Flammulina* spp.). There are many other fungi like milk mushrooms, morels, chanterelles, truffles, black trumpets and porcini mushrooms (*Boletus edulis,* also known as 'king boletes') (Ghorai, 2009).

Many worldwide cultures, especially in the Orient, recognize that extracts from some edible and non-edible mushrooms are known for their potential health benefits. In China, the dietary supplements and nutraceuticals made from mushroom extracts are used, along with various combinations of other herbal preparations (Barros *et al*., 2008*a*; Carbonero, 2006).

These mushrooms have attracted attention because they are source of non-starchy carbohydrates, with a high content of dietary fiber (chitimous wall), moderate quantities of proteins (20-30% of dry matter) with most of the essential amino acids, minerals and vitamins (B) (Ghorai, 2009, Agrahar-Murugkar and Subbulakshmi, 2005).

Several compounds with important pharmaceutical properties have been isolated from these organisms. Substances that act as anti-aging, in longevity, modulating the immune system, having hypoglycemic activity and to inhibit tumor growth have been isolated from mushrooms, such as polysaccharides. Polysaccharides can interconnect several points forming a wide variety of branched or linear structures, for example, ß-

glucans (Ooi and Liu, 2000). The structural variability of polysaccharides shows regulatory mechanisms with various interactions in higher organisms (Carbonero, 2006, Agrahar-Murugkar and Subbulakshmi, 2005). Furthermore, other bioactive substances such as triterpenes, lipids and phenols have also been identified and characterized in mushrooms with medicinal properties (Maiti, 2008).

Fungi can be produced technically through fermentative process. The media may be the available substrates from valued cheap sources like agro-biomass and industrial wastes; transformed into high value added food and pharmaceutical products.

2 Biological Properties of Mushrooms

2.1 Antioxidants Activity

Exogenous chemical and endogenous metabolic process in the human body or in the food system might produce highly reactive free radicals, especially oxygen derived radicals, which are capable of oxidizing biomolecules, resulting in cell death and tissue damage (Elmastas *et al*., 2007). During the reduction of molecular oxygen, reactive oxygen species (ROS) are formed and there is a continuous requirement for inactivation of these free radicals. Superoxide and hydroxyl radicals are the two most representative free radicals. In cellular oxidation reactions, superoxide radical is normally formed first, and its effects can be magnified because it produces other kinds of cell-damaging free radicals and oxidizing agents. Damage induced by free radicals can affect many biological molecules, including lipids, proteins, carbohydrates and vitamins present in food. Reactive oxygen species also implicate in the pathogenesis of various human diseases, such as DNA damage, carcinogenesis, rheumatoid arthritis, cirrhosis, arteriosclerosis as well as in degenerative processes associated with ageing. Evidences have been indicating that diet rich in antioxidant reduce risks of some diseases (Elmastas *et al*., 2007; Liu *et al*., 1997).

Mushroom contain vitamins A and C of ß-carotene and a great variety of secondary metabolites such as phenolics compounds, polyketides, terpenes, steroids and phenols, all have protective effects because of their antioxidant properties (Jayakumar *et al.*, 2009; Soares *et al*., 2009).

Researchers investigate several edible and non-edible mushrooms with antioxidant properties for applications in food, cosmetics and treatment of diseases (Table 1). Water or ethanolic extracts of fruiting bodies or biomass resulting by fermentation have been studied and tested.

Table 1: Potential antioxidant of mushrooms

Mushroom	Antioxidant Activity		
	Sources	**Substances**	**References**
Agaricus bisporus	Fruit body	Methanolic extracts	Elmastas *et al*., 2007
Agaricus brasiliensis	Fruit body	Methanolic extracts	Soares *et al*., 2009
Agaricus silvaticus	Fruit body	Methanolic extracts	Barros *et al*., 2008b
Agrocybe cylindracea	Fruit body	Ethanolic and hot water extracts	Tsai *et al*., 2007
Antrodia camphorata	Submerged Fermentation	Methanolic extracts	Shu and Lung, 2008
Boletus edulis	Fruit body	Hot water extracts	Ribeiro *et al*., 2008
Boletus edulis	Fruit body	Alkaloids	Sarikurkcu *et al*. 2008
Boletus badius	Fruit body	Methanolic extracts	Elmastas, *et al*., 2007
Cordyceps sinensis	Submerged Fermentation	Polysaccharide	Leung *et al*., 2009
Geastrum saccatum	Fruit body	Glucans	Dore *et al*., 2007
Grifola frondosa	Fruit body	Water extracts	Lee *et al*, 2008b
Hypsizigus marmoreus	Fruit body	Cold and Hot water, Ethanolic extracts	Lee *et al*., 2007
Inonotus obliquus	Fruit body	Methanolic extracts	Lee *et al*., 2007b
Laetiporus sulphureus	Fruit body	Ethanolic extracts	Turkoglu *et al*., 2007
Lentinula edodes	Fruit body	Ethanolic extracts	Zheng *et al*., 2005
Leucopaxillus giganteus	Fruit body	Methanolic extracts	Barros *et al*. 2008b
Lepista nuda	Fruit body	Methanolic extracts	Elmastas *et al*., 2007
Phellinus linteus	Fruit body	Ethanolic extracts	Song *et al*., 2003
Pleurotus ostreatus	Fruit body	Methanolic extracts	Elmastas *et al*., 2007
Pleurotus ostreatus	Fruit body	Ethanolic extracts	Jayakumar Thomas and Geraldine, 2009
Polyporus squamosus	Fruit body	Methanolic extracts	Elmastas *et al*., 2007
Russula delica	Fruit body	Methanolic extracts	Elmastas *et al*., 2007
Suillus collitinus	Fruit body	Methanolic extracts	Sarikurkcu Tepe and Yamac, 2008
Turbinaria conoids	Fruit body	Fucoidan	Chattopadhyay *et al*., 2009
Verpa conica	Fruit body	Methanolic extracts	Elmastas *et al*., 2007

Mushrooms are currently available in Taiwan, including *Agaricus blazei*, *Agrocybe cylindracea* and *Boletus edulis*. Ethanolic extracts, usually, were more

effective than hot water in antioxidant activity. However, for analyses of scavenging ability on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, hot water extracts were more effective in reducing power, scavenging ability on hydroxyl radicals, and chelating ability on ferrous ions (Tsai *et al*., 2008). Sarikurkcu *et al.* (2008) studied the antioxidant activity of *Lacttarius deterrimus*, *Suillus collitinus*, *Boletus edulis* and *Xerocomus chrysenteron*. The species of *L. deterrimus* and *B. edulis* showed activities as strong as the positive controls. The reducing power of the species was excellent. Chelating capacity of the extracts was proportional to the increasing concentration.

Hypsizigus marmoreus (peck) Bigelow, also known bunashimeji and hon-shimeji is a mushroom cultivated and commercially available in Taiwan. Naturally occurring antioxidants components, including tocopherols and total phenols, were found in extracts from *H. mamoreus*. The major antioxidant components found in hot water extracts were total phenols and in ethanolic extracts were total tocopherols (Lee *et al*., 2008a).

Northeast of Portugal is recognized as one of the richest regions of Europe in wild edible mushrooms species, which have considerable gastronomic relevance. *Russula cyanoxantha*, *Amanita rubescens*, *Suillus granulates* and *Boletus edulis* are among more common and marketed species. Caps and stipes of four species were studied and showed rich in organic acids, but phenolics compounds are present in low amounts. Organic acids, phenolics compounds and alkaloids composition were insufficient to justify the antioxidant potential of analyzed species. Other compounds also participate in the observed activity. These species present antioxidant potential, especially high for *Boletus edulis*, which is also the richest specie in alkaloids (Ribeiro *et al*., 2008). *Boletus edulis* was a popular edible mushroom in Europe, North America and Asia. *Clitocybe maxima*, *Pleurotus ferulae* and *P. ostreatus* were used to study antioxidant properties. Ethanolic extracts and hot water extracts from *P. ferulae* and *P. ostreatus* were more effective than *C. maxima* cap and stipe antioxidants activities. Total phenols were major naturally occurring antioxidant components found in the range of 5.51-9.66 gallic acid equivalents (GAE)/g and 5.10-11.1mg gallic acid equivalents/g for ethanolic and hot water extracts, respectively. These mushrooms could be used in grams levels as food or food ingredient. Therefore, these three mushrooms might serve as possible protective agents in human diets to help human reduce oxidative

damage (Tsai *et al.*, 2008). Metabolisms, physiology of mushroom and environmental conditions are important and determinant to the production of antioxidant compounds. However, the extraction methodologies are primordial to remove intracellular compounds and/or others substances resulting from metabolism (Table 2). The antioxidant activity of five mushroom species: *Agaricus bisporus*, *Agaricus arvensis* Schaeffer, *Agaricus romagnesi* Wasser, *Agaricus silvaticus* Schaeffer and *Agaricus silvicola* were analyzed. All the species tested to have antioxidant activity, especially *A. silvaticus* (Barros *et al.*, 2008b). Soares *et al.* (2009) investigated the young and mature extracts of fruiting bodies of *Agaricus brasiliensis*. Both extracts showed antioxidants activities, except the chelating ability of ferrous ions. Consumption of fruiting bodies of *A. brasiliensis* might be beneficial to the human antioxidant protection system against oxidative damage. Extraction use polar and non-polar solvents, considering solubility and thermostability of each substance, respectively (Table 2).

Table 2: Contents of total phenols in extracts from mushrooms

Mushrooms	Total phenols of extracts (mg/g)			References
	Hot Water	Ethanolic	Methanolic	
Agaricus arvensis	-	-	2,72	Barros *et al.*, 2008b
Agaricus bisporus	-	-	4,49	Barros *et al.*, 2008b
Agaricus romagnesii	-	-	6,18	Barros *et al.*, 2008b
Agaricus silvaticus	-	-	8,95	Barros *et al.*, 2008b
Agaricus silvicola	-	-	6,45	Barros *et al.*, 2008b
Agaricus blazei	5,67	5,80	-	Tsai *et al.*, 2007
Agrocybe cylindracea	5,6	5,7	-	Tsai *et al.*, 2007
Boletus edulis	5,81	5,73	-	Tsai *et al.*, 2007
Hypsizigus marmoreus	10,01	6,89	-	Lee *et al.*, 2008
Clitocybe maxima (cap)	9,71	9,66	-	Tsai *et al.*, 2008
Clitocybe maxima (stipe)	5,1	5,51	-	Tsai *et al.*, 2008
Pleurotus ferulae	7,73	6,71	-	Tsai *et al.*, 2008
Pleurotus ostreatus	11,1	7,11	-	Tsai *et al.*, 2008
Pleurotus ostreatus	-	5,49		Jayakumar *et al.*, 2009

Shiitake (*Lentinus edodes*) have several compounds including bioactive polysaccharides (lentinan), dietary fiber, ergosterol, vitamin B_1, B_2 and C and minerals have been isolated from de fruiting body, mycelia and culture medium of this mushroom. Heat treatment of Shiitake sample increased the overall content of free polyphenolic and flavonoid compounds. The heat treatment can produce changes in their extractability due to disruption of the cell wall thus bound polyphenolic and flavonoid compounds may be released more easily relative to those of raw materials (Choi *et al.*, 2006).

The ethanolic extract of the *P. ostreatus* showed concentration-dependent antioxidants activities by inhibiting lipid peroxidation, scavenging hydroxyl and superoxide radicals, reducing power and chelating ferrous ions when compared to different standards such as ascorbic acid, butylated hydroxytoluene (BHT) and ethylenediamine tetraacetic acid (EDTA) (Jayakumar *et al.*, 2009).

2.2 Anti-inflammatory Activity

Inflammatory response is succession of cellular reactions involving the generation and release of cellular mediators such as cytokines. The excessive amount or duration of the production of cytokines, especially TNF-α can cause serious harm to the body (Dudhgaonkar, Thyagaranjan and Sliva, 2009).

Excessive or unregulated production of these mediators has been implicated in mediating a number of diseases including rheumatoid arthritis, osteoarthritis, sepsis, chronic pulmonary inflammatory disease, Crohn's disease, ulcerative colitis, and also carcinogenesis. Inflammation is inherent to pathogenesis of a variety of diseases. Inhibition of activation and the proliferation of these inflammatory cells appears to be an important therapeutic target for some drugs in the treatment of inflammatory diseases and cancer (Dudhgaonkar, Thyagaranjan and Sliva, 2009; Van *et al.*, 2009; Wu *et al.*, 2008a).

TNF-α is a major pro-inflammatory cytokine with diverse biological activities. Large quantities of TNF-α may induce intravascular thrombosis, shock and cachexia.

Macrophages play important role in host-defense mechanism, and inflammation. The overproduction of inflammatory mediators by macrophages has been implicated in

several inflammatory diseases and cancer. The activation of macrophages is important in the investigation of defensive response such as the production of interleukins IL-1ß, IL-6, TNF-α, reactive oxygen species, prostaglandin and nitric oxide. IL-1 ß are also a multifunctional cytokine, which has been implicated in pain, fever, inflammation and autoimmune conditions. It stimulates acute phase protein synthesis in the liver and may cause rise in body temperature. It is also up-regulated in many inflammatory diseases. IL-6 is a multifunctional cytokine with pro-/anti-inflammatory properties. Nitric oxide is an important messenger in diverse pathological functions, including neuronal transmission, vascular relaxation, immune modulation and cytotoxicity against tumor cells. Nitric oxide, secretor product of mammalian cells, is produced by inducible nitric oxide synthase, endothelial nitric oxide synthase, and neuronal nitric oxide synthase is considered an important signaling molecule in inflammation (Van *et al*., 2009). Mushrooms have been applied in the treatment of infections in popular culture or medicine in many countries, such as China, Japan, Russia and Brazil. The anti-inflammatory properties of mushrooms have interested researchers and motivated the investigation of some species (Table 3).

Table 3: Potential Anti-inflammatory of Mushrooms

Mushrooms	Anti-inflammatory Activity		
	Sources	**Substances**	**References**
Agrocybe cylindracea	Fruit body	Agrocybin	Ngai, Zhao and Ng, 2005
Amanita muscaria	Fruit body	Hot water, methanolic and ethanolic extracts	Michelot and Melendez-Howell, 2003
Fomitopsis pinicola	Submerged fermentation	Polysaccharides	Cheng *et al*., 2008
Ganoderma lucidum	Fruit body	Triterpene	Dudhgaonkar *et al.,* 2009
Geastrum saccatum	Fruit body	Glucans	Guerra Dore *et al*., 2007
Inonotus obliquus	Fruit body	Hot water extracts	Van *et al.,* 2009
Phellinus linteus	Fruit body	Butanol fraction	Kim *et al*., 2004
Poria cocos	Submerged fermentation	Polysaccharides	Lu *et al*., 2009
Pleurotus nebrodensis	Fruit body	Nebrodolysin	Lv *et al*., 2009
Pleurotus pulmonarius	Fruit body	Polysaccharides	Smirdele *et al*., 2008

The *Geastrum saccatum,* a mushroom native from Brazil, is produced under natural conditions in the unexplored reserve of "Mata da Estrela-Rio Grande do Norte". This basiodiomycete is a saprobiotic fungus and it is well adapted to tropical regions. The mushroom, known as "Star of the Land", is used in popular medicine by

obstetricians and healers, and has curative properties for eye infections and diseases, such as asthma. In the anti-inflammatory effects of glucans *G. saccatum* on carragennan-induced pleurisy and was observed that the glucans decreased the number of cells from pleural fluid rats. There is evidence that inhibition of nitric oxide synthase reduces the production of prostaglandins by cicllooxigenase through reduced synthesis of oxide nitric, these decrease several inflammatory symptoms such as vessel dilation. Thus, it could be related to inhibition of diapedesis of cells as mononuclear leukocytes in the inflammation site when used the glucans. The animals treated with glucans decreased oxide nitric. These effects suggest an anti-inflammatory effect of glucans *G. saccatum* (Dore *et al*., 2007).

Lu *et al*., (2009) demonstrated that *Poria cocos*, called Fu Ling in China, can participate in the regulation of the anti-inflammatory process. *P. cocos* are commercially available and are popularly used in the formulation of nutraceuticals, tea supplements, cosmetics, and functional foods in Asia. Chemical compounds found in *P. cocos* include triterpenes and ß-pachyman, a polysaccharide composed of ß-pachimarose, pachymic acid, and poricoic acid. IFN is one of the major mediators which predispose endothelial cells toward inflammatory/immunological responses. The pre-treatment with the polysaccharide extracted of *P. cocos* was dose-dependently and inhibited IFN induced inflammatory gene IP-10 protein release. It suggests that the effect of polysaccharide on IP-10 expression was regulated at the translational level and thus it may participate in regulating inflammatory-related diseases. This polysaccharide showed no toxicity to endothelials cells, indicating the safety of its use.

Inonotus obliquus also known as Chaga, is a black mushroom that grows on birch trees in northern climates such as in Russia. These mushrooms act as traditional medicine to treat gastrointestinal cancer, cardiovascular disease and diabetes. Polyphenolic compounds produced by Chaga can protect cells against oxidative stress. It also showed to inhibit platelet adhesion and aggregation, which plays an important role in thrombosis. Those platelets are important in hemostasis and modulation of the inflammatory response, including the released of cytokines, Chaga may be involved in various aspects in the inflammatory. Levels of nitrite, which is an indicator of oxide nitric concentration, displayed a significant decline when treated with Chaga. The inflammatory effect caused by Chaga may be a cascade effect with inhibition of oxide nitric production (Van *et al*., 2009).

Dudhgaonkar and researchers (2009) showed that triterpene extract from medicinal mushroom *Ganoderma lucidum* markedly suppressed in the inflammatory response in LPS-active murine macrophages. Specifically, triterpene by *G. lucidum* suppressed LPS-dependent secretion of TNF-α, IL-6, oxide nitric and prostaglandin E2 from murine macrophages cells. The inhibition of production of oxide nitric and prostaglandin E2 by *G. lucidum* triterpene was mediated through the down-regulation of expression of induction nitric oxide synthase and ciclooxigenase-2, respectively. Moreover, this triterpene inhibited LPS-dependent induction of NF-κB as well as expression, phosphorylation and nuclear translocation of p65 NF-κB subunit. Also, triterpene of *G. lucidum* seem to be potent in suppressing the key molecules responsible in the inflammatory response. Extract of *G. lucidum* containing triterpenes or isolated triterpenes (ganoderic acid A, F, DM, T-Q, lucidenic acid A, D_2, E_2, P, methyllcidenate A, D_2, E_2, Q and 20-hydroxylucidenic acid N) suppressed ear-edema inflammation in laboratories animals.

Phellinus linteus, traditional mushroom medicine in oriental countries, showed topical anti-inflammatory activity. Extract ethanolic was evaluated using croton oil-induced ear edema test and showed an inhibitory effect on inflammation. Among the subfractions, the butanol fraction appeared to be most effective in anti-inflammation, supposing that *P. linteus* have hydrophilic compounds (Kim *et al*., 2004).

Curiously, *Amanita muscaria* is not considerable edible mushroom, but has been used for various purposes, mostly as a psycostimulant, by different ethnic groups from Mexico, Siberia and Eastern Asia. Slavic nations have their own traditions of *A. muscaria* use. Ethnic people are especially fond of the beneficial effects they achieve from topical application of ethanolic extract (or strong vodka). Hot water, methanolic and ethanolic extracts of *A. muscaria* have description of their use for reduction of the consequences of inflammatory processes in cases of rheumatic diseases, body injuries, insect bites, others (Michelot and Melendez-Howell, 2003).

2.3 Antimicrobial Activity

Antibiotic agents have been effective usually therapeutic since their discovery in the 20th century. However, it has paradoxically resulted in the emergence and dissemination of multi-drug and resistant pathogens. Antibiotic resistance represents a prospect of therapeutic failure for life-saving treatments (Hearst *et al.*, 2009).

The search for new drugs is necessary for infectious diseases treatment and to be able to inhibit growth resistant bacteria. Biologist and others researches related that some mushrooms need special attention in natural environments because of the relation with other species, local growing, conditions of temperature, substrates and others environmental factors. Mushrooms interestingly showed antimicrobial activities, some examples are table 4.

Table 4: Potential antimicrobial of fruit body of mushrooms

Antimicrobial Activity		
Mushrooms	**Substances**	**References**
Ganoderma japonicum	Essential oil	Liu *et al.*, 2009
Ganoderma lucidum	Water extracts	Wu *et al.*, 2006b
Ganoderma lucidum	Ganodermin	Wang and Ng, 2006
Laetiporus sulphureus	Ethanol extracts	Turkoglu *et al.*, 2007
Lentinula edodes	Water extracts	Hearst *et al.*, 2009
Lentinula edodes	Chloroform extract	Hirasawa *et al.*, 1999
Leucopaxillus giganteus	Methanol	Barros *et al.*, 2008b
Pleurotus sajor-caju	Ribonuclease	Ngai and Ng, 2004
Russula delica	Ethanol extracts	Yaltirak *et al.*, 2009
Russula paludosa	Lacase	Wang, Wang and Ng, 2007
Tricholoma giganteum	Trichogin	Guo, Wang and Ng, 2005

Laetiporus sulphureus (Bull.) Murrill is a wood-roting basidiomycete, growing on several tree species and producing shelf-shaped fruit-bodies of pink-orange colour, except for the fleshy margin which is bright yellow. *Laetiporus* species contain N-methylated tyramine derivatives, polysaccharides, a number of lanostane, triterpenoids, laetiporic acids and other metabolites have reported that might have potential as food colorants. The antimicrobial effect of ethanol extracts of *L. sulphureus* was tested against six species of Gram-positive bacteria, seven species of Gram-negative bacteria and one species of yeast. The most susceptible bacterium was *Micrococcus flavus* with

23 ± 1 mm diameter of the halo of inhibition. The ethanol extract of *L. sulphureus* showed no antibacterial activity against *Klebsiella pneumonie* at the concentration used. The ethanol extract exhibited high anticandidal activity on *Candida albicans* (Turkoglu *et al*., 2007).

Russula delica Fr. is used as food in Turkey and grows under coniferous and deciduous trees. The antimicrobial effect of ethanolic extract of *R. delica* was tested against three species of Gram positive bacteria and six species of Gram negative bacteria. Results showed inhibitory activity against *Shigella sonnei* and *Yersinia enterocolitica*. Natural antimicrobials agents are safest to the people and present low risk for resistance development by pathogenic microorganisms (Yaltirak *et al*., 2009).

Hirasawa *et al.* (1999) studied the antibacterial activity of shiitake extracts (*Lentinula edodes*) as a preventive agent against dental caries and adult periodontitis. Shiitake extracts were antibacterial effective against *Streptococcus* spp., *Actinomyces* spp., *Lactobacillus* spp., *Prevotella* spp., and *Porphymonas* spp., of oral origin. This extract of mushroom can be used to prevent dental caries and periodontitis because also supports the idea that inhibit the formation of water-insoluble glucans from sucrose by glucosyltransferase.

Rao, Millar and Moore (2009) studied the activities of shiitake freeze-dried powder. Bioassay of the extracts showed that all the fractions exhibited qualitative inhibitory activity against bacteria and fungi. Thirty-four compounds from extracts of the shiitake was identified, for example: Cycloheximide (antibiotic that acts as a plant growth regulator, but causes human liver toxicity and reduction in protein synthesis); Bostrycoidin (bioactive *in vitro* against *Mycobacterium tuberculosis*); Anticarcinogenic alkaloids (muscarine, choline); Tanins (epiafzelechin); Terpenoids (adiantone); Cyclopiazonic acid (a natural food contaminant); Aspergillomarasmine; Disulphides, lenthionine compounds in the organic extracts.

Shiitake mushroom extract had extensive antimicrobial activity against 85% of the organisms tested, including 50% of the yeast and mould species in the trial. These results were compared with the positive control (Ciprofloxacin) and Oyster mushroom, considering the number species inhibited by the activity of the metabolites inherent to the shiitake mushroom (Hearst *et al*., 2009).

Mushroom proteins have important play antimicrobial activity. Mushroom compositions have contained 2–40% protein according with species. Each protein of

mushroom has specific sequence of amino acids and molecular weight. Antifungal proteins have function of protecting organisms from the deleterious consequences of fungal assault; they display a spectacular diversity of structures. An antifungal peptide with a molecular mass of 9 KDa was isolated from fresh fruiting bodies of the mushroom *Agrocybe cylindracea*. The antifungal peptide, designated as agrocybin, exhibited remarkable homology to RPI 3, a cysteine-rich-protein that is expressed during fruiting initiation in *Agrocybe chaxingu*. Agrocybin is also similar to grape (*Vitis vinifera*) antifungal peptide in N-terminal sequence. Data suggest that antifungal function of agrocybin is important during fruiting initiation for protecting fruiting bodies. Agrocybin inhibits mycelial growth in *Mycosphaerella arachidicola*, in line with the majority of fungal proteins and peptides that exert their antifungal action against a number of fungal species and is not effective against a variety of bacteria (Ngai, Zhao and Ng, 2005).

Pleurotus nebrodensis produce hemolysin that can be implicated as a virulence factor. This hemolysin was named nebrodolysin and showed antiviral activity, besides it exhibits a suppressive action on HIV-1 and reproducible antiviral effect. The mechanism of the antiviral suggested that nebrodeolysin might act in a different way by interaction the infection of the virus (Lv *et al*., 2009).

Antifungal protein from *Ganoderma lucidum*, ganodermin, inhibits mycelial growth in the phytopatogenic fungi *Botrytis cinerea*, *Fusarium oxysporum* and *Physalospora piricola* (Wang and Ng, 2007).

Niohshimeji (*Tricholoma giganteum*), produced trichogin, antifungal proteins monomeric and have N-terminal sequence. This protein showed antifungal activity against *F. oxysporum*, *M. arachidicola* and *P. piricola*. The antifungal activity of protein this mushroom is high compared with others antifungal proteins. Trichogin inhibits HIV-1 reverse transcriptase (Guo, Wang and Ng, 2005).

Ngai and Ng (2004) demonstrated antimicrobial activity of ribonuclease from the extract of *Pleurotus sajor-caju*. The ribonuclease inhibited mycelial growth in the fungi *Fusarium oxysporum* and *M. arachidicola* and bacteria as *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The molecular mass of *P. sajor-caju* RNase is 12 KDa and poly U-specific and high activity, compared with others mushrooms. The N-terminal sequence of *P. sajor-caju* RNase bears resemblance to the terminal sequence of a bacteriocin peptide. And it showed a sequence of the two enzymes involved in

RNA-specific editase. This structural feature of *P. sajor-caju* RNase may be related to its antibacterial and RNase activities. *Tricholoma giganteum* produces lacase that is characterized with N-terminal sequence dissimilar from reported N-terminal sequences of mushroom lacases. Its molecular mass (43 kDa) is smaller than most of the reported mushroom lacases which are around 60kDa (Wang and Ng, 2004)

 Russula paludosa is a wild edible mushroom collected from Chine. Its fruiting bodies are abundant in the summer. Extracts from fruiting bodies of *R. paludosa* exhibited an inhibitory effect on HIV-1 (Wang, Wang and Ng, 2007).

 Ganoderma japonicum is found in China and has been used for the treatment of various diseases. The essential oil of *G. japonicum* has pharmacologicals effects and contains bactericidal components, such as (E)-nerolidol, linalool and (2E, 4E)-decadienal. The antimicrobial results indicated that oil inhibited mainly Methicillin-resistant *Staphylococcus aureus* (MRSA). This component has been confirmed to have bacteriostatic and bactericidal activity, causing changes in cell membrane permeability and bacterial death (Liu *et al*., 2009).

2.4 Antitumoral Activity

 The National Cancer Institute (US National Institutes of Health) define cancer as an abnormal growth of cells which tend to proliferate in an uncontrolled way and, in some cases, to metastasize. Cancer cells can spread to other parts of the body through the blood and lymph systems. There are more than 100 different types of cancer. Cancer types can be grouped in main categories (Table 5):

Table 5: Classification of cancer types

Categories	Definition
Carcinoma	Begins in the skin or in tissues that line or cover internal organs
Sarcoma	Begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue
Leukemia	Starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood
Lymphoma and myeloma	Begin in the cells of the immune system
Central nervous system cancers	Begin in the tissues of the brain and spinal cord

Source: http://www.cancer.gov - September, 2009

According to World Health Organization (WHO) related lung, stomach, liver, colon and breast cancer cause the most cancer deaths each year. About 30% of cancer deaths can be prevented. It is estimated that in 2030 there will be 26 million cases of cancer worldwide. Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030 (http://www.who.int/en/).

International Union Against Cancer (IUAC) reported that Africa less than 0.1% and Asia is only 8.5% of the population is covered by cancer registration. The Chernobyl disaster was a nuclear reactor accident that occurred on 26 April 1986 at the Chernobyl Nuclear Power Plant in Ukraine and it is now estimated that by 2065 there will be 16000 cases of thyroid cancer and 28000 cases of other cancers in Europe as a result of this accident (http://www.uicc.org/). Several mushroom species are studied and purified substances such as polysaccharides and proteo-polysaccharides are recognized to be the potent immunomodulatory and antitumor (Table 6).

Table 6: Potential antitumoral of mushrooms

Mushrooms	Antitumoral Activity		
	Sources	Substances	References
Agaricus brasiliensis	Submerged fermentation	Polysaccharides	Fan *et al*., 2007
Agrocybe aegerita	Fruit body	Methanolic extracts	Diyabalanage *et al*., 2008
Albatrellus confluens	-	Grifolin	Ye *et al*., 2007
Cordyceps sinensis	Submerged fermentation	Polysaccharides	Yang *et al*., 2005
Coriolus versicolor	Fruit body	Terpenoids and polyphenols	Harhaji *et al*., 2008
Fomes fomentarius	Fruit body	Polysaccharides	Chen *et al*., 2008
Ganoderma capense	Fruit body	Lectin	Ngai and Ng, 2004
Ganoderma lucidum	Solid fermentation	Polysaccharides	Rubel *et al*., 2008
Ganoderma tsugae	Submerged fermentation	Polysaccharides	Peng *et al.,* 2005
Grifola frondosa	Submerged fermentation	Polysaccharides	Cui *et al*., 2007
Inonotus obliquus	Submerged fermentation	Polysaccharides	Kim *et al*., 2006
Lentinula edodes	Fruit body	Fiber	Choi *et al*., 2006
Lentinula edodes	Fruit body	Polysaccharides	Frank *et al*., 2006
Lentinula edodes	Fruit body	Ethanolic	Hatvani, 2001
Poria cocos	Submerged fermentation	Polysaccharides	Huang *et al*., 2007
Pleurotus citrinopileatus	Fruit body	Lectin	Li *et al*., 2008
Pleurotus ostreatus	Fruit body	Methanolic	Tsai *et al*., 2008

A. blazei Murril also called *A. brasiliensis* is native to southern Brazil, popularly known as "Himematsuke" in Japan, or "Cogumelo do Sol" in Brazil. Consumption has increased in Brazil, Japan, Korea, Canada and United States because of its medicinal properties. Mechanism studies have demonstrated that antitumor activities of *A. blazei* extracts can be related to induction of apoptosis, cell-cycle arrest and inhibition of tumor-induced, neovascularization, immunopotentiation and restoration of tumor-suppressed host immune system.

The exopolysaccharide produced by *A. brasiliensis* showed strong inhibition against Sarcoma 180. The complete regression ratio was 50% and the suppression ratio percentage was 72.19%. Exopolysaccharide was characterized a mannan-protein

complex, with its molecular weight being 10^5–10^7 kDa by gel filtration and contained of glucose, galactose and ribulose (Fan *et al*., 2007).

Kim *et al*., (2009) optimized the extraction of *A. blazei* for isolation of bioactive compounds with antitumor effects. Extracts of mushroom was obtained with different polarities and solubilities. One fraction, extracted at 80^oC using 70% water-ethanol (v/v) showed tumor inhibitory activity against the human promyelotic leukemia cells *in vitro*.

Lee *et al*. (2008c) showed dietary intake of *A. blazei* Murril fed at 6250, 12500 and 25000 ppm for two years of dry powder appears to enhance survival in males and not appear to be carcinogenic in rats.

Grifolin is a natural, biologically active substance isolated from fresh fruiting bodies of the mushroom *Albatrellus confluens*. Studies showed that grifolin is able to inhibit the growth of some cancer cell lines *in vitro* by induction of apoptosis. Ye *et al*. (2007) showed that grifolin inhibits the proliferation of nasopharyngeal carcinoma cell line through G1 phase arrest, which mediated by regulation of G1-related protein.

Cordyceps militaris is the best-known and most frequently collected bug-killing *Cordyceps*, but there are dozens of "entomogenous" species in North America (Kuo, 2006).

Park *et al*. (2009) demonstrated that water extracts of *C. militaris* may increase mitochondrial dysfunction, and results in the activation of caspase-9, leading to the activation of caspase-3 target proteins. The caspase family proteins are known to be one of the key executioners of apoptosis. Water extracts of *C. militaris* induces apoptosis in human lung carcinoma cells.

Coriolus versicolor, also known as Yun-Zhi, produces bioactive compounds. Hot water and ethanol extracts from *C. versicolor* demonstrated activities antitumoral to treatment of melanoma cells. According to analysis, the predominant compounds are terpenoids and polyphenols. The prevention of tumor growth was exerted through diverse mechanism including cell cycle suspend, induction of tumor cell death by apoptosis and secondary necrosis, together with stimulation of the anti-tumor activity of macrophages (Harhaji *et al*., 2008).

Peng and collaborators (2005) also studied *C. versicolor* and showed inhibitory effect on the growth of Sarcoma 180 solid tumors. Anti-tumor activity of polysaccharo-peptide resides in its anti-angiogenic properties, via a suppression of vascular

endothelial growth factor gene expression, resulting in a deprivation of angiogenic stimulation to the tumor growth.

Ganoderma lucidum is known as "mushroom of immortality" because enhancing longevity. Researchers have also demonstrated that *G. lucidum* inhibits the migration of breast cancer cells and prostate cancer cells, suggesting the reduction tumor invasiveness. Since integrins are the major cell surface adhesion molecules expressed by all cell types. Tests showed that incubation with *G. lucidum* polysaccharides reduced integrin expression. Integrins are composed of α and ß transmembrane subunits. Each α and ß combinations has its own binding specificity and signaling properties (Wu *et al.*, 2006).

G. lucidum polysaccharides used dose-dependently treatment enhance catalase activity in the polysaccharides-treatment groups when compared to the ovarian cancer model (YouGuo, Zongli and XiaoPing, 2009).

Fruiting body of *Ganoderma tsugae* is used to promote health and longevity in Orientals countries. Peng *et al.*, (2003) related that results indicate that ß-D-galacto-α-D-mannan isolated from culture filtrate of *G. tsugae* mycelium also exhibited significant antitumor activities. In 2005, the same researchers demonstrated anti-tumor activities were observed in three polysaccharides fractions with inhibition ratio 50%.

Mushrooms with antitumoral properties are controversy for some researchers. Studies showed collateral effects caused by mushrooms. Sadava *et al.* (2009) analyzed cytotoxicity of twelve species of *Ganoderma* and just four species showed non-cytotoxic effects. However, active *Ganoderma* extracts induced apoptosis.

Grifola frondosa, has been reported to posses many biologically active compounds. Especially, the antitumor and immune-stimulating activities of polysaccharide D-fraction, a branched ß-(1-6)-D-glucan isolated from the fruiting body. Most reports conformed that mushroom polysaccharides exerted their antitumor action via activation on the immune response of the host organism, and mushroom polysaccharides were regarded as biological response modifiers. Polysaccharides from different strains have different antitumor activities *in vitro*. Data suggests that the polysaccharides fractions from *G. frondosa* had selective antitumor activities on the different tumor cell lines (Cui *et al.*, 2007).

Surenjav *et al.* (2006), studied lentinan, (1-3)-ß-D-glucan, an antitumor polysaccharide, has been isolated from the fruiting body of *Lentinus edodes*. The triple helical (1-3)-ß-D-glucan antitumor containing protein showed activities against the growth of Sarcoma 180. The triple helical conformation plays an important role in the enhancement of the antitumor activities. Data suggesting that the antitumor activity of polysaccharide is also related to their molecular weight and content of the bound protein.

Li *et al.* (2008b) describe antitumoral activity of *Hedysarum polybotrys* Hand.-Maz (HP). In China, is used in the treatment of diseases, such as cancer, glycemy and immunomodulatory, anti-aging, anti-oxidation activities. The α-(1$\rightarrow$4) - D-glucan showed that inhibit proliferation of human hepatocellular carcinoma and human gastric cancer.

Inonotus obliquus is a white rot fungus, called Chaga, is a medicinal mushroom that has been used in Siberian and Russia folk medicine to treat stomach discomforts. Extracts of *I. obliquus* are known to inhibit the growth and protein synthesis of tumor cells. Alpha-linked-fucoglucomannan isolated from cultivated mycelia of *I. obliquus* can inhibit tumor growth *in vivo*. The endopolysaccharide-mediated inhibition of tumor growth is apparently caused by an induced humoral immunity of the host defense system rather than by a direct cytotoxic effect against tumor cells (Kim *et al.*, 2006).

Huang *et al.*, (2007) studied *Poria cocos* called Fu Ling, it is collected between July and September in China. Polysaccharides fractions was tested and showed strong inhibition against leukemia cell proliferation at all concentrations. The three water-soluble fractions presented significantly high inhibition ratio of more 80% at concentration of 200 µg/mL.

Phellinus ignarius, an orange color mushroom is used to improve health and remedy various diseases, such as gastroenteric disorders, lymphatic diseases and cancer. Extracts from fruiting body of *P. igniarus* inhibited the proliferation of SK-Hep 1 cells and RHE cells in a concentration-dependent manner, with IC_{50} values of 72 and 103 µg/mL (Song *et al.*, 2008).

Pleurotus is an important mushroom because it has gastronomic, nutritional, commercial and medicinal properties. *P. citrinopileatus* is a widely used edible mushroom, delicious taste and rich in nutrients. Antitumor activities of lecitin from *P.*

citrinopileatus are similar to those *P. ostreatus* lecitin. *P. citrinopileatus* lecitin is dimeric, like lecitins from others mushrooms (Li *et al.*, 2008c). Sarangi *et al.* (2006) demonstrated that two fractions of *P. ostreatus* can directly kill Sarcoma 180 cells *in vitro*. Cell-cell adhesion determines the polarity of cells and participates in the maintenance of the cell societies called tissues. Adhesion is generally reduced in human cancer cells in the treatment with extracts of mushroom. Reduced intercellular adhesion allows cancer cells to disobey the social order, resulting in the destruction of histological structure, which is the morphological hallmark of malignant tumors. Reduced intercellular adhesiveness is also indispensable for cancer invasion and metastasis. Tong *et al.* (2009) observed also antitumor activity against HeLa tumor cell *in vitro*, in a dose-dependent manner and exhibited lower cytotoxicity to human embryo kidney cells.

Leucopaxillus giganteus is enormous mushroom is often found growing in large fairy rings or arcs in woodland clearings. It is apparently widely distributed, but most common in the Pacific Northwest and Rocky Mountains (Kuo, 2006).

Ren *et al.* (2008) demonstrated that clitocine isolated from *L. giganteus* have proliferation inhibitory activity against HeLa cells in a dose-dependent manner by mechanism involved the induction of apoptosis.

2.5 Immunomodulatory Activity

Molecules derived of the secondary metabolism macrofungi are known by medicinal properties. For example, polysaccharides and glycoproteins are involved in the innate and adaptive immunity, resulting in the production of cytokines. The therapeutic effects of these compounds such as antitumor and anti-infective activity and suppression of autoimmune diseases have been associated in many cases with their immunomodulating effects (Table 7).

Compounds that are capable of interacting with the immune system to up regulate or down regulate specific aspects of the host response can be classified as immunomodulators or biologic response modifiers. These agents can be applied in treating and preventing diseases (Moradeli *et al.*, 2007). Innate immunity serves as an essential first line of defense against microbial pathogens and may also influence the

nature of the subsequent adaptive immune response. Phagocytic cells, such as macrophages and neutrophils, play a key role in innate immunity because of their ability to recognize, and destroy many pathogens by oxidative and non-oxidative mechanisms. Bioactive polysaccharides and polysaccharide-protein complexes have been isolated from mushrooms (Table 7), yeast, algae, lichens and plants, and these compounds have attracted significant attention because of their immunomodulatory and antitumor effects (Xie, Schepetkin and Quinn, 2007). Mushroom polymers have immunotherapeutic properties by facilitating growth inhibition and destruction of tumors cells. Fungal ß-glucans-induced immune responses are different in their actions in immune therapies based on supplementation of elements of the immune system and stimulating the immune system, can be option in treatment of diseases. These compounds are not synthesized by humans and inducing both innate and adaptive immune response (Chen and Seviour, 2007). Proteoglycans and polysaccharides have high molecular mass, constituted of β-glucans, cannot penetrate cells, so the first step in the modulation of cellular activity is binding to immune cell receptors. The mechanism by which the innate immune system recognizes and responds to compounds of mushroom is complex and multi-factorial process. After this activation and signaling occurs the humoral- and cell-mediate immunity induction (Moradali *et al*., 2007).

Table 7: Potential immunomodulatory activity

Immunomodulator Activity			
Mushrooms	**Sources**	**Extractions/comp ounds**	**References**
Agaricus blazei	Fruit body	Water soluble compounds	Kasai *et al*., 2004
Agaricus blazei	Fruit body/mycelium	Biocompounds	Shimizu *et al*., 2002
Coriolus versicolor	Submerged fermentation	Polysaccharide	Lee *et al*., 2006
Ganoderma lucidum	-	Fractions	Ji *et al*., 2007
Ganoderma lucidum	-	Polysaccharide	Zhu *et al*., 2007
Grifola frondosa	Submerged fermentation	Polysaccharide	Yang *et al*., 2007
Grifola frondosa	Submerged fermentation	Polysaccharide	Wu *et al*., 2006
Inonotus obliquus	Submerged fermentation	Polysaccharide	Kim *et al*., 2006
Lentinula edodes	Fruit body	Polysaccharide	Gu and Belury, 2005
Lentinula edodes	Fruit body	Polysaccharide	Zheng *et al*., 2005

Extracts of *A. blazei* from the fruiting body and the mycelium were effective in activation of the human complement pathway. Both bioactive compounds have been

demonstrated to be potent activators of the complement system in human serum in a dose and time dependent manner (Shimizu *et al.*, 2002).

Kasai *et al.* (2004) analyzed *A. blazei* fraction induced expression of IL-12, a cytokine known to be a critical regulator of cellular immune responses. According to Kimura *et al.* (2006), supplementation of *A. blazei* was effective in the activation of enzymes related to energetic metabolism in leukocytes of calves. *A. blazei* extract was water-soluble and easy to deal with as a food additive.

Lentinan, schizophyllan and krestin have been accepted as immunoceuticals in several oriental countries. Increase of natural killer cells, cytotoxic T lymphocytes and delayed type hypersensitivity responses against tumor antigen were observed after administration of lentinan. *Lentinula edodes* was claimed to have a range of health benefits. The concentration of TNF-α, IFN in serum increased significantly in the polysaccharide groups compared with the model control group. Also, polysaccharide of *L. edodes* increased oxide nitric production in peritoneal macrophages and catalase activity of macrophage (Zheng *et al.*, 2005).

Polysaccharopeptides produced for *C. versicolor* can stimulate cytokines production but also demonstrated that a critical time for culture harvesting is essential for obtaining optimal bioactivities of the fungi (Lee *et al.*, 2006).

Ganoderma lucidum is a Chinese medicinal fungus, which has been clinically used in East Asia and is given considerable attention for treatment for various diseases. Medicinal functions have been assigned to crude extracts and isolated components of *G. lucidum.* The potential immunomodulating activity is the capacity of a particular substance to influence specific immune functions such as activating individual components of the immune system and promoting cytokine synthesis. Ji *et al.* (2007) showed that extracts of *G. lucidum* activated mouse macrophages in a dose dependent manner, increased the levels of IL-1, IL-12p35 and IL-12p40 gene expression, and significantly enhanced oxide nitric production. These immunomodulatory functions suggest that *G. lucidum* may also interfere with the growth of certain tumors.

Bao *et al.* (2001) demonstrated that (1-3)-D-Glucans of *G. lucidum* have immunomodulating and antitumor activities. The structural and physicochemical properties and lymphocyte proliferation activity of all samples varied with the functionalized groups and the degree of substitution. The results of immunological assays indicated that some modified derivatives had stimulating effects on lymphocyte

proliferation and antibody production and the introduction of carboxymethyl group with low degree of substitution was the best choice on the improvement of the immunostimulating activity.

G. lucidum mycelia stimulated moderate levels of TNF, IL-6 and IFN- release in human whole blood and moderately stimulate cytokine production without potentiating oxide nitric release. The ineffectiveness in inducing oxide nitric release by *G. lucidum* mycelia indicates that the compositions and structures of glucan in mycelia and fruiting body may be different, and this might result in enhancing innate immune response through different receptors or pathways (Kuo *et al.*, 2006).

G. lucidum polysaccharides enhanced the activity of immunological effectors cells in immunosuppressed mice and promoted phagocytosis and cytotoxicity of macrophages. The above beneficial effects induced by the low-dose of polysaccharide treatment did not result in any side effects (Zhu *et al.*, 2007).

Polysaccharides obtained from fermented and fruiting body of *Grifola frondosa* have demonstrated many interesting biological activities. Exopolymers fractions of *G. frondosa* can be enhancers of innate response and considered as potent materials for immune system (Shi *et. al.*, 2008; Yang *et al.*, 2007).

Grifolan, polysaccharide of *G. frondosa,* showed that hot water-soluble fractions (polysaccharides) of mycelia from submerged fermentation can effectively induce innate immunity and therefore enhance pro-inflammatory cytokine release, phagocytosis, and Natural Killer cytotoxicity activity *in vitro* (Wu *et al.*, 2006).

Inonotus obliquus is a white rot fungus widely distributed over Europe, Asia, and North America. The polysaccharide yield of species of *Inonotus* increased proportionally with an increasing cell mass of the fermentation broth. However, the immunostimulating activities were not proportional to the corresponding polysaccharide yield. High specific activities of endopolysaccharide were obtained during both the late lag and the late stationary phases, but not during the active cell growth phase, indicating that the polysaccharide activity is probably closely related to cell age. During the late lag phase, low total activities were obtained due to low cell masses in spite of high specific activities. The specific activity of endopolysaccharide at late lag phase appeared to be highly similar to the activity at late stationary phase. The endopolysaccharide of *I. obliquus* showed much higher splenic cell activities than the corresponding exopolysaccharide (Kim *et al.*, 2006).

2.6 Anti-angiogenic Activity

Angiogenesis can be characterized as an integrate set of cellular, biochemical and molecular processes in which new blood vessels are formed from pre-existing vessels. This occurs physiologically during reproductive and developmental processes as well as during the late phases of wound healing following tissue damage (Contois, Akalu and Brooks, 2009). Blood vessels run through every organ in the body (except cornea and cartilage), assuring metabolic homeostasis by supplying oxygen and nutrients and removing waste products. Therefore, angiogenesis is known to be essential in several physiologic processes, such as organ growth and development, wound healing and post-ischemic tissue repair. However, inappropriate or aberrant angiogenesis contributes to the development and progression of various pathological conditions including tumor growth and metastasis, diabetic retinopathy, cardiovascular diseases, inflammatory disease and psoriasis. Angiogenesis can be separated into several main steps, such as degradation of the basement membrane of exiting blood vessels, migration, proliferation and rearrangement of endothelial cells, and formation of new blood vessels (Makrilia *et al.*, 2009; Ramjaum and Hodivala-Dilke, 2009; Ribatti, 2009).

This switch clearly involves more than simple up-regulation of angiogenic activity and is known to be the result of net balance between positive and negative regulators. There are three particularly important stimulators of angiogenesis: i) vascular endothelial growth factor ii) fibroblast growth factor; iii) angiopoetins; between many others, like platelet derived growth factor, epidermal growth factor, ephrins; transforming growth factors alpha and beta, interleukins, chemokines, and small molecules, such as sphingosine 1-phosphate, that are known to promote cell proliferation, survival and differentiation of endothelial cell (Jung *et al.*, 2008; Duarte, Longatto Filho and Schmitt, 2007; Stupack, Storgard and Cheresh, 1999).

Anti-angiogenesis strategies are based on inhibition of endothelial cell proliferation, interference with endothelial cell adhesion and migration, and interference with metalloproteases. Down-regulation of angiogenesis has been considered to be advantageous for the prevention of tumors (Bhat and Singh, 2008; Jung *et al.*, 2008; Song *et al.*, 2003).

In Taiwan, the mushroom *Antrodia cinnamomea* is known as "niu-cha-ku" or "chang-chih" and produces triterpene acids, steroid acids and polysaccharides with biological activities. The fraction >100 kDa of polysaccharide from *A. cinnamomea* showed potential anti-angiogenic *in vivo* and *ex vivo* indirectly by immunomodulation (Table 8) (Yang *et al.*, 2009).

Table 8: Anti-angiogenesis activity of mushrooms

Mushrooms	Anti-angiogenesis Activity		
	Sources	Extractions/compounds	References
Antrodia cinnamomea	Mycelia	Hot water extracts	Yang *et al.*, 2009
Ganoderma tsugae	Fruit body	Methanol extracts	Hsu *et al.*, 2009
Grifola frondosa	Fruit body	Water extracts	Lee *et al*, 2008b
Fomitopsis pinicola	Fruit body	Polysaccharides	Cheng *et al.*, 2008
Phellinus linteus	Fruit body	Ethanolic extracts	Song *et al.*, 2003

Ganoderma sp contains numerous bioactive natural components and the two categories of those are polysaccharides and triterpenoids, both of them are potent inhibitors of *in vitro* and *in vivo* tumor growth. The epidermal growth factor receptor activation is often linked with angiogenesis. Methanol extracts of *Ganoderma tsugae* showed antiangiogenic effects on the cancer cells by the downregulation of vascular endothelial growth factor (Hsu *et al.*, 2009).

Fomitopsis pinicola is marketed as a tea and food supplement. Chemical compounds found in *F. pinicola* include steroids, sesquiterpenes, lanostane triterpenoids and triterpene glycosides. Ethanolic extracts and polysaccharides of *F. pinicola* were effective for the anti-angiogenesis at 10 µg/mL concentration and showed no toxicity up to concentration of 1 mg/mL (Cheng *et al.*, 2008).

Grifola frondosa demonstrated anti-angiogenic activity by inhibit vascular endothelial growth factor induced angiogenesis *in vivo* and *in vitro.* Vascular endothelial growth factor is the most angiogenic factor associated with inflammatory diseases and cancer (Lee *et al.*, 2008b). Ethanolic extracts from *Phellinus linteus* presented anti-angiogenic activity and can be used as adjuvant chemotherapy for the treatment of cancer (Song *et al.*, 2003)

2.7 Hypoglycemic Activity

The World Health Organization describes diabetes as a chronic condition that occurs when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin that is it produced. Hyperglycemia and other related disturbances in the body's metabolism can lead to serious damage to many body's systems, especially to the nerves and blood vessels. Diabetes is a life threatening condition. World Health Organization indicates that worldwide almost 3 million deaths per year are attributing to diabetes.

Roglic *et al*. (2005) describe that excess mortality attributed to diabetes accounted for 2–3% of deaths in poorest countries and over 8% in the U.S., Canada, and the Middle East. In people 35–64 years old, 6–27% of deaths were attributable to diabetes.

Functional foods and naturals compounds have become a popular approach to prevent occurrence of diabetes mellitus. Several mushroom species have been described to have anti-diabetic properties, because were found compounds as fibers source, polysaccharides and other biological activities (Table 9).

Table 9: Hypoglycemic activity of mushrooms

	Hypoglycemic Activity		
Mushrooms	**Sources**	**Extractions/compounds**	**References**
Cordyceps sinensis	Fruit body	Polysaccharide	Li *et al*., 2006
Coprinus comatus	Submerged fermentation	Vanadium	Han *et al*., 2006
Marasmius androsaceus	Mycelia	Ethanol extracts	Zhang *et al*., 2009
Phellinus baumii	Submerged fermentation	Polysaccharide	Hwang *et al*., 2005
Sparassis crispa	Fruit body	Powder	Kwon *et al*., 2009
Tremella mesenterica	Submerged fermentation and fruit body	Fruit body Water extracts	Lo *et al*, 2006

Vanadium compounds have the ability to imitate action of insulin. Oral administration of inorganic vanadium salts, have shown anti-diabetic activity *in vitro*, *in vivo* and even in patients. Vanadium at lower doses (0.18 mg/kg/d) was absorbed by

fermented mushroom of *Coprinus comatus*, which is one rare edible fungus that is able to absorb and accumulate trace elements. *C. comatus* is a munshroom claimed to benefit glycemic control in diabetes and others properties. *C. comatus*, on a dry weight basis, contains, on the average, 58.8% carbohydrate, 25.4% protein and 3.3% fats, with the rest constituted of minerals (Table 9). It indicates that *C. comatus* could supplement nutrients to the mice as well as lower blood glucose of hyperglycemic mice. *C. comatus* has the ability to take up and accumulate trace metals. However, the toxicity associated with vanadium limits is therapeutic efficacy (Han *et al.*, 2006).

Tremella mesenterica contains up 20% of polysaccharide glucuronoxylomannan in the fruiting bodies, is a popular, edible and medicinal mushroom in orient. Fruiting bodies, submerged fermentation and the acid polysaccharide of *T. mesenterica* have anti-hyperglycemic activity. Consumption of *T. mesenterica* by rats had improved short- and long-term glycemic responses, as evidence by significantly decreased blood glucose concentrations in oral tolerance test and serum fructosamine concentrations, respectively. This mushroom has potential anti-hyperglicemic functional food or nutraceuticals for diabetes with daily ingestion of 1 g/Kg of fruiting bodies, biomass and glucuronoxylomannan. Consumption of *T. mesenterica* may act functional food in improving the short and long-term glycemic control in persons with high risk of diabetes or type 2 diabetes mellitus, not in persons with type 1 diabetes mellitus (Lo *et al.*, 2006).

Phellinus baumii is a mushroom used as a folk medicine for a variety of human diseases in several Asian countries. The plasma glucose level in the exopolysaccharide-fed rats was substantially reduced by 52.3% as compared to the diabetic rats, which is the highest hypoglycemic effect among mushroom-derived. The activities of alanine aminotransferase and asparate aminotransferase were decreased by administration of *P. baumii* exopolysaccharide, thereby exhibiting a remedial role in liver function. *P. baumii* of exopolysaccharide administration led to the diabetogenic effect and significantly reduced the degree of diabetes. Oral administration of *P. baumii* of exopolysaccharide may have a potential benefit in preventing diabetes, since pancreatic damage induced by environmental chemicals and other factors are cause of diabetes (Hwang *et al.*, 2005).

Exopolysaccharides of *G. lucidum* (Lingzhi) have hypoglycemic effects. Studies showed that treatment with water extract of *G. lucidum* for 4-week oral gavages, 0.3

g/kg for consumption, lowered the plasma glucose level in mice. Phosphoenolpyruvate carboxykinase is a hepatic enzyme which is important in the regulation of gluconeogenesis. Inhibition of the hepatic phosphoenolpyruvate carboxykinase reduced blood glucose levels and improved glucose tolerance together with a decreased circulating free fatty acid and triacylglycerol levels in the diabetic mice. *G. lucidum* consumption caused a suppression of the hepatic phosphoenolpyruvate carboxykinase gene expression with a concomitant reduction of the serum glucose levels in mice (Seto *et al*., 2009).

Cordyceps, one of the most valued traditional Chinese medicines grow on caterpillar. Polysaccharide from *Cordyceps* protects against free radical induced neuronal cell toxicity. Polysaccharides of *Cordyceps* produced a drop in blood glucose level in both normal and diabetic animals, at doses of higher than 200 mg/kg. Hypoglycemic effect is possibly because of the increase in blood insulin level, which may be due to the induced insulin release from the residual pancreatic cells and/or reduced insulin metabolism in body by polysaccharide (Leung *et al*., 2009; Li *et al*., 2006a).

Lima *et al*. (2008) showed that diet supplemented with exopolysaccharides of *A. brasiliensis* provided during 8 weeks to the mice reduced in glucose plasma concentration around 22%. It has been also demonstrated in another study that the supplementation with β-glucans and oligosaccharides obtained from the fruiting body of *A. brasiliensis* reduced in the glucose serum concentration in rats. Mushroom could have an anti-diabetic activity by promoting the insulin release by the Langerhans cells in the pancreas. The total cholesterol ratio in the mice was reduced 27% with *A. brasiliensis* exopolysaccharide supplementation. Fruiting body biomass and the mushroom polysaccharides have significant anti-hyperglycemic activity and the abilities to increase glucose metabolism and insulin secretion in type 2 diabetes mellitus. However, the mechanisms of action for the exopolysaccharide of *A. brasiliensis* on cholesterol and glucose metabolism are still unknown.

Mechanisms which contribute to the formation of free radicals in diabetes include non-enzymatic and auto-oxidative glycosylation, metabolic stress resulting from changes in energy metabolism, levels of inflammatory mediators, and the status of antioxidant defense. Selective damage of islet cells in the pancreas may be one of the pathological mechanisms for Type I diabetes. Antioxidants could prevent the

development of diabetes. Polysaccharide also has a strong antioxidant property which can protect cultured rat pheochromocytoma cells from being damaged by hydrogen peroxide. This antioxidant activity of polysaccharide may also play a protective role in the development of diabetes (Li *et al*, 2006a).

Kwon *et al*. (2009), described consequences caused for diabetes, such as difficult proliferation of cells, decreased collagen production, decreased chemotaxis and phagocytosis, reduction in the levels of growth factors, and the inhibition of fibroblast proliferation. *Sparassis crispa,* a medicinal mushroom consumed in China and Japan was investigated for diabetes treatment. In experiments, the diabetes was induced and was accompanied by diabetic symptoms such as weight loss, polyuria, hyperglycemia, and neuroendocrine dysfunction. The oral administration of *S. crispa* demonstrated positive effects, such as: increased migration of macrophages and fibroblasts, collagen regeneration, and epithelialization under hyperglycemic conditions.

2.8 Anti-hypertensive Activity

According to World Health Organization (WHO), cardiovascular diseases include coronary heart disease (heart attacks), cerebrovascular disease, raised blood pressure (hypertension), peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. The major causes of cardiovascular disease are tobacco use, physical inactivity, and an unhealthy diet. Hypertension management and risk prediction based on diastolic blood pressure may be reasonably valuable for younger people. The use of diastolic blood pressure as main treatment has been supported by the discovery that essential hypertension is characterized by increased peripheral vascular resistance and raised mean arterial pressure, which more closely correlates with diastolic blood pressure than with systolic blood pressure (Ono, Oyekigho and Adeleke, 2006).

Angiotensin, an oligopeptide in the blood, causes vasoconstriction and increased blood pressure. It is part of the renin-angiotensin system, which is a major target for drugs that lower blood pressure. Angiotensin II may cause the narrowing of the vessels and increases the pressure, resulting in high blood pressure (hypertension). Angiotensin II is formed from angiotensin I in the blood by the enzyme angiotensin converting enzyme. Angiotensin I converting enzyme is potentially of great importance for

controlling blood pressure in the rennin-angiotensin system. The rennin-angiotensin system plays an important role in interrelated set of mechanism for the control of the volume, pressure and electrolyte composition of blood. Angiotensin I converting enzyme converts the inactive decapeptide angiotensin I to potent vessel pressure octopepetide angiotensin II, the main active component (Hagiwara *et al*., 2005; Lima, 1998).

Hatakeshimeji mushroom (*Lyophyllum decastes* Sing.), which have a delicious taste, forms a family of Honshimeji (*Lyophyllum shimeji*) and belongs to a highly related genus of *Lyophyllum*. Hot water-extracts of Hatakeshimeji inhibited angiotensin converting enzyme activity in rats (Kokean *et al*., 2005). For consume of mushrooms and preserve biological activities, cooking method choice, without causing the destruction of both physiological effects and the taste, is important. Kokean *et al*. (2005) studied the influence of cooking and processing. The results of deep-frying test did not adversely affect the taste, the texture and the antihypertensive property of Hatakeshimeji (Table 10).

Table10: Hypocholesterolemic Activity of mushrooms

Hypocholesterolemic Activity			
Mushrooms	**Sources**	**Extractions/compounds**	**References**
Auricularia auricular	Fruit body	Powder	Cheung, 1996
Pleurotus ostreatus	Mycelia	Proteo-glucan	Sarangi *et al*., 2006
Pleurotus ostreatus	Fruit body	Dried Fruit body	Bobek *et al*., 1998
Tremella fuciformes	Fruit body	Powder	Cheung, 1996
Activity Anti-hypertensive			
Lyophyllum decastes (Sing.)	Fruit body	Powder and water extracts	Korean *et al*., 2005
Pleurotus nebrodensis	Fruit body	Polysaccharide and water extracts	Miyazawa, Okazaki and Ohga, 2008

Tamogi-take (*Pleurotus cornupiae*) is an edible mushroom that belongs to Hiratake family and grows on standing and fallen elm trees on the Siberian peninsula of Russia and in the eastern and northem parts of Hokkaido, Japan. This mushroom produced D-mannitol, which inhibits angiotensin converting enzyme activities and lowers the blood pressure of spontaneously hypertensive rats. The compound was identified as D-mannitol by direct comparison of spectral data with authentic compound. The inhibition of Angiotensin I converting enzyme is dose-dependent by

various sugars. D-mannitol, D-sorbitol and D-dulcitol are classified as sugar alcohols (IC_{50}:16.4 mM) and were the most effective inhibitor of Angiotensin I converting enzyme. Although sugar alcohol might prevent an increased in blood pressure by mechanism such as osmotic diuretic effect, more studies are necessary to explain the mechanism *in vivo* (Hagiwara *et al.*; 2005).

Pleurotus nebrodensis is native from Southern Europe, Central Asia and China, and have been shown to prevent hypertension. Compounds of *P. nebrodensis* were administered orally, and antihypertensive actions were measuring blood pressure. Two hours after administration, the blood pressure decreased around 85% and increased gradually after 6 until 48 hours pos-administration (Miyazawa, Okazaki and Ohga, 2008).

Marasmius androsaceus, a traditional chinese mushroom, is usually used in tendon relaxation, pain alleviation and anti-hypertension. The 3,3,5,5 Tetramethyl-4-piperidone is an active compound prepared from *M. androsaceus* but, the action unclear. Study showed that 3,3,5,5 Tetramethyl-4-piperidone have effects reducing blood pressure in anesthetic tested in rats and cats. It can inhibit the automatic rhythmic contraction of ileum section in guinea pig. It also can inhibit the concentration of rabbit aorta smooth muscle caused by adrenalin. 3,3,5,5 Tetramethyl-4-piperidone has a simple structure with low molecular weight, which is suitable to serve as leading compound. This is an antihypertensive compound, and the effect is partially related to ganglionic blocking (Zhang *et al.*, 2009).

2.9 Prebiotics

Prebiotics are ''selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health''. The effect of a prebiotic is indirect because it selectively feeds one or a limited number of microorganisms thus causing a selective modification of the host's intestinal microflora. Intestinal bacteria leads towards a consideration of factors that may influence the flora composition in a manner than can impact upon health (Reid, 2008; Vasiljevic and Shah, 2008). Some criteria must be considered in order to be defined as prebiotic are: resistance to the upper gut tract,

fermentation by intestinal microbiota, beneficial to the host health, selective stimulation of probiotics, stability to food processing treatments (Wang, 2009).

Ingestion of prebiotic was believed to enhance immune function, improve colonic integrity, decrease incidence and duration of intestinal infections, down-regulated allergic response as well as improve digestion and elimination of faeces (Aida *et al*., 2009).

Digestibility and bioavailability of mushroom constituents have been missing from the knowledge of mushroom nutritional value. The dry matter of mushroom fruit bodies is about 5-15%, they have a low fat content and contain 19-35% proteins. The content of carbohydrates, which are mainly present as polysaccharides or glycoproteins, ranges 50-90%. Most abundant mushroom polysaccharides are chitin, hemicelluloses, ß- and α-glucans, mannans, xylans and galactans. Mushroom is potential candidate for prebiotics as it contains carbohydrates like as linear and branched glucans with different types of glycosidic linkages, such as (1-3), (1-6)-ß-glucans and (1-3)-α-glucans, but some are heteroglicans containing glucorinic acid, xylose, galactose, mannose, arabinose or ribose (Table 11). Like polysaccharides originated from other food products, they contribute to the digestion process as soluble or insoluble dietary fibers depending on their molecular structure and conformation. ß-glucans are recognized as immunological activators and are effective in treating diseases like cancer, diabetes, hypercholesterolemia and others. Chitin is a water-insoluble structural polysaccharide, accounting for up to 80–90% of dry matter in mushroom cell walls. A high proportion of indigestible chitin apparently limits availability of other components (Aida *et al*., 2009; Kalac, 2009; Synytsya *et al*., 2009).

Table 11: Biological Activity of mushrooms

Mushrooms	Sources	Extractions/compounds	References
Biosynthesis of collagen			
Grifola frondosa	Submerged Fermentation	Polysaccharides	Lee *et al*., 2003
Activity Mitogenic			
Pleurotus citrinopileatus	Fruit body	Lectin	Li *et al*., 2008
Activity Prebiotic			
Pleurotus ostreatus	Fruit body	Glucans	Synytsya *et al*., 2009

Synytsya *et al.* (2009) studied extract of *Pleurotus ostreatus* and *Pleurotus eryngii* with potential prebiotic activity. Specific soluble glucans were isolated from mushrooms by boiling water and alkali extraction. Potential prebiotic activity of aqueous extracts and alkali extracts was tested using nine probiotic strains of *Lactobacillus*, *Bifidobacterium* and *Enterococcus*. The utilization of both extracts by different manner confirms different chemical structures of polysaccharides, such as prebiotics. Extracts of *Pleurotus* can be used for symbiotic construction with selecting probiotic strains.

3.0 Conclusion

Fruit bodies of mushrooms are traditionally used in oriental countries like food and several diseases treatment. Mushrooms may be beneficial to people with impaired immune systems, diabetes, infectious diseases, hypertension, heart diseases, cancer and others. The mechanisms of action of these compounds from mushrooms still not completely understand. Responses to such bioactive compounds are to be mediated by cell-surface receptors and cell-cell interactions, which may be present only on specific subsets of cells. Studies are necessary to explain the mechanism these compounds: optimization of the production process, chemical identification and quantification of specific compounds responsible for potential benefit, receptors cells. Higher fungi produce biological compounds, mainly in secondary phase of metabolism. The submerged fermentation is an alternative process for bioactive compounds production and industrial application. The science widens opportunity of progress in the development of new treatments, by discovering natural compounds with different activities and applications.

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Chapter II

PRODUCTION OF EXOPOLISACCHARIDES OF MUSHROOM BY SUBMERGED FERMENTATION

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1 Introduction

Mushrooms are macroscopic fungi, known for medicinal properties and its attractive potential application in pharmaceuticals industries. There are approximately about 140.000 different species of mushrooms in world, however, only about 10% are known and close to 70 species present some pharmacological properties (Moradali *et al.*, 2007, Sicoli *et al.*, 2005, Wasser *et al.*, 2002).

Different compounds are produced by mushrooms and are used alternative therapies for human, especially in oriental countries. Several medicinal properties are influenced by mushrooms, such as immunological enhancement, maintenance of homeostasis and regulation of biorhythm. They have also been used in the prevention and treatment of several diseases, such as cancer of the stomach, esophagus, lungs, cerebral stroke, arteriosclerosis, diabetes, heart diseases, etc (Kitzberger *et al.*, 2007; Elmastas *et al.*, 2006; Shon and Nam, 2001; Mizuno *et al.*, 1999).

The constituent molecules of Macromycetes organelles and secondary metabolites are known as bioactive compounds such as polysaccharides, glycoproteins or proteoglycans, terpenoids, fatty acids, proteins, lectins, etc. Mushroom produces exopolysaccharides and researches identify frequently a ß-D-glucan compound. ß-D-glucan belongs to group glucose polymers whose monomers are linked by ß-glycosidic bound, which give to glucan its structural function. Their molecular weight, degree of branching, and nature of the branches are believed to determine their bioactive and functional effects (Pérez-Guisado, 2007).

In traditional methods of mushroom production are used substrates of agricultural and industrial wastes, which can be transformed into mushroom fruit body. The fungal inoculum is added to a pasteurized substrate in growing containers, and usually grown in the dark in climate-controlled rooms. The fruit bodies of mushrooms happen in six weeks after spawning. This method present difficult the application of the pest management programs, particularly for diseases. And according to the substrate there is an influence the in centesimal composition and nutritional value of mushroom (Cui *et al.* 2006).

The submerged fermentation is an interesting alternative for the safety production of bioproducts with clinical or pharmacological interest. Submerged culture of mushroom for production of exopolysaccharide (EPS) has received attention, because of their beneficial biological activities (Kim *et al.*, 2009; Tong *et al.*, 2009; Youguo, Zongji and Xiaoping, 2009; Li *et al.*, 2008; Ren *et al.*, 2008; Lee, Yen and Mau, 2007; Jung *et al.*, 2007; Lim and Yun, 2006). Submerged fermentation of mushroom was conducted since 1948, reported by Humfeld and subsequently developed by Humfeld (1948) and Sugihara (1952) to permit it low-cost and large-scale production. Block *et al.* (1953) published that a strain of *Agaricus blazei* was adapted to submerged culture indicating the continuous interest of this method of mycelium propagating. They reported excellent yields of mushroom mycelium grown on orange juice, citrus press water, and chemically defined media. However, the major objective was to produce flavor and taste of mushrooms.

Submerged fermentation gives rise to potential advantages of higher mycelial production in a compact space and shorter time without significant contamination risk, availability of convenient control, high product concentration and easy downstream processing (Komura *et al.*, 2010; Tang *et al.*, 2008; Shih *et al.*, 2008; Lim and Yun, 2006, Wu *et al.*, 2006).

Mushroom differs in structure, composition and physiological activity. Each species of mushroom, according to the culture conditions, can produce bioactive compounds, which are similar to those found in fruit bodies (Cui *et al.*, 2006; Kim *et al.*, 2002). The optimization of exopolysaccharide production and mycelial biomass of mushroom involves the study of nutritional factors, such as carbon, nitrogen source and environmental conditions, such as pH, temperature, aeration, pre-inoculum (Komura *et al.*, 2010, Lee *et al.*, 2007).

The aim of this review was to present the polysaccharides importance and some parameters of the submerged fermentation of mushrooms for exopolysaccharides production.

2 Polysaccharides of Mushroom

Non-cellulosic ß-glucans of mushroom consist of a backbone of glucose residues usually joined by ß-(1-3) linkages (Chen and Sevoiur, 2007; Dalmo and Bogwald, 2008).

Polysaccharides may have several chemical structures, such as ß-glucans, heteroglucans, heteroglycan, ß-glucan-protein, α-manno-ß-glucan, α-glucan-protein and heteroglycan-protein complexes (Zhang, 2007, Chen and Sevoiur, 2007).

ß-glucans are majority constituents of cell wall, named endopolysaccharides or also can be excreted into culture medium, exopolysaccharide. These compounds are interesting biological activities, such as: stimulating immune system, antitumoral activity, potential in treating liver diseases, diabetes, prebiotics, hypercholesterolemia, anti-aging, longevity promoter and others (Table 1). Structural features such as (1→3)-ß-linkages in main chain the glucan and additional (1→6)-ß-branch points have been indicated as important factors in antitumor action. ß-glucans containing mainly 1→6 linkages exhibit less activity, possibly due their inherent flexibility of having too many possible conformations. The primary structure of polysaccharide is defined by the monosaccharide composition and sequence, and by the position of glycosidic linkages, as well as the nature, number and location of appendices no-carbohydrate groups (Chen and Seviour, 2007; Zhang *et al.*, 2007).

(1→3)-ß-glucans from mushroom have antitumoral activity. Studies suggest that activity of polysaccharides depends of structural conformation, size, and molecular weight. The proposed mechanisms by which mushroom polysaccharide inhibit cell cancer growth are: i) prevention by consume of polysaccharides; ii) stimulating immune system; iii) direct activity (Zhang *et al.*, 2007). There is study that ß-glucans are recognized by cell-surface receptors, for example: complement receptor 3, lactoylceramide, scavenger receptors, Dectin-1 and tol like receptor (Chen and Sevoiur, 2007, Firenzouli, Gori and Lombardo, 2008). Fruit bodies were better studied than the product obtained by submerged fermentation. Compounds produced by submerged fermentation was researched and compared with fruiting bodies compounds of mushrooms.

Some therapeutic effects were analyzed to optimize the production processes (Table 1).

Table 1: Biological activities of EPS produced by Macromycetes

EPS extraction by fruit body of mushroom			
Mushrooms	**Biological Activity**	**Composition of EPS**	**References**
Agaricus brasiliensis	Antitumoral	EPS	Pinto *et al.*, 2009
Cordyceps militaris	Anti-inflammatory	Man, xyl, rham and gal	Yu *et al.*, 2004
Cordyceps sinensis	Antitumoral	α-D-glucan	Wu *et al.*, 2007
Ganoderma atrum	Antioxidant	Man, gal and glc	Chen *et al.*, 2008
Ganoderma lucidum	Antitumoral	EPS	YouGuo, ZongJi, XiaoPing, 2009
Ganoderma lucidum	Immunomodulatory	Glcp and galp	Bao *et al.*, 2002
Geastrum saccatum	Antioxidant, anti-inflammatory	ß-glucan	Guerra Dore *et al.*, 2007
Lentinus edodes	Antitumoral	(1-3)-ß-D-glucan	Zhang *et al.*, 2005
Lentinus edodes	Antitumoral	(1-3)-ß-glucan	Surenjav *et al.*, 2006
Pleurotus ostreatus	Immunomodulatory	(1-6)-α-D-galactopyranosyl	Carbonero, 2006
Pleurotus ostreatus	Antitumoral	Man, Gal, Glc	Tong *et al.*, 2009
Pleutorus pulmonaris	Anti-inflammatory and analgesic	(1-3)-β-D-glucopyranosyl	Smirdele *et al.*, 2008
EPS Extraction by mycelium of mushroom			
Antrodia cinnamomea	Anti-angiogenic	EPS >100 KDa	Yang *et al.*, 2009
Grifola Frondosa	Antitumoral	Glucan	Nie *et al.*, 2006
Poria cocos	Anti-inflammatory	Galactan	Lu *et al.*, 2009
EPS production by submerged fermentation of mushroom			
Coriolus versicolor	Immunomodulatory and antitumoral	Polysaccharo-peptides	Lee, Yang and Wan, 2006
Fomitopsis pinicola	Antiangiogenic	Fuc, gal, glc, man, fru	Cheng *et al.*, 2008
Pleurotus tuber-regium	Antitumoral	ß-glucan	Zhang *et al.*, 2005
Grifola frondosa	Antitumoral	Galactopyranose and glucopyranose	Cui *et al.*, 2007
Hericium erinaceus	Immunological	Mannan	Lee, Chon and Hong, 2009
Phellinus baumii	Anti-diabetic	EPS	Cho *et al.*, 2006
Tremella fuciformis	Anti-diabetic	EPS	Cho *et al.*, 2006

According to Kim *et al*, (2002), EPS was obtained from submerged fermentation and exhibited the similar biological effect than those intrapolysaccharide in mycelia.

3 Parameters of Fermentation

As the production of polysaccharide from mycelia is more efficient than from fruit body, the influence of culture conditions from submerged cultures of mushroom presented an interesting alternative. Various investigators have pointed out that both culture and environmental conditions affect the production and physical-chemical characteristics of EPS. The effects of various carbon and nitrogen sources, their concentrations, initial pH and fermentation duration on the production of mycelia in terms of dry weight, EPS and inner polysaccharide (IPS) by mushrooms can affect the mycelial growth and EPS production rate, and the EPS productivity (Lee, Yen and Mau., 2007, Pokhrel and Ohga, 2007, Wu *et al*., 2006).

3.1 Culture Media

Few attempts have been made to obtain optimal submerged culture conditions for polysaccharide production from Macromycetes. However, studies on nutritional requirements and environmental conditions in submerged fermentation investigate the optimal media culture for each strain. Researchers have tested alternatives to increase the production of microbial metabolites using some stimulating agents, including fatty acids, surfactants, vegetable oils, and organics solvents. These stimulation agents are known to mediate cell permeability by disorganizing the cell membrane and/or directly affecting the level of enzyme synthesis involved in product formation, thereby contributing to enhanced production of the target products (Shi *et al.*, 2008; Lim and Yun, 2006).

3.2 Carbon Source

Carbohydrates are a major component of the cell cytoskeleton and are a key nutritional requirement for growth and development of mushroom in submerged fermentation. It is necessary to find carbon sources for the best bioactive molecule production.

The sugar compositions of EPS varied with the carbon sources. Possibly, different carbon sources might have different effects of catabolic repression on the cellular secondary metabolic (Lee *et al.*, 2007).

Each mushroom has a physiologic response with different carbon sources. For example in the submerged cultivation of *Cordyceps militaris*, a low cell density and growth rate was observed when lactose was used as carbon source, whereas the cell growth in galactose medium was higher (Mao and Zhong, 2006).

Agaricus brasiliensis showed the highest production of EPS when sucrose was used, however cell growth was not favored. In a medium containing glucose concentration was exhausted at initial phase with 20 and 30g/L. Results suggest that a relativity higher initial glucose concentration led to higher production, productivity and yield of EPS (Zou, 2006).

The growth pattern of *Pleurotus tuber-regium* in the media with monosaccharides, fructose was preferred than with glucose by the mycelium. This was probably due to the more efficient incorporation of fructose directly in the respiratory pathway after phosphorylation (Wu *et al.*, 2003).

Experiments with *Lyophyllum decastes* demonstrated that glucose is clearly a good carbon source for EPS production in submerged cultures of mushroom. Growth and other metabolic activities could happen in a specific concentration of glucose. In addition, the maximum mycelial growth was accompanied by a higher production of polysaccharides. Glucose has been reported as a good respiratory substrate. However, this demonstration showed that each carbon source was independently responsible for mycelial growth and polysaccharide production (Pokhrel and Ohga, 2007).

Tang and Zhong (2002) found that lactose was the best carbon source while sucrose was the worst from the viewpoint of cell growth of *Ganoderma lucidum*. These indicate that the utilization of sources varies among the mushrooms.

Tang *et al*., (2008) reported the kinetics of EPS accumulation under different carbon source. *Tuber sinense* cells did not growth in the presence of lactose. However, the highest EPS production, of 1.61 ± 0.40 g/L was obtained. Results indicate that lactose was favorable for EPS production and productivity (Table 2).

Different carbon sources may have different effects of catabolic repression on the cellular secondary metabolism (Kim et al., 2005).

Tang and Zhong (2002) reported that many short mycelia were observed by microscope when sucrose was used as carbon source. A poor cell growth and a higher release of polysaccharide to the medium may be related with the morphological change. The osmotic pressure caused by high sugar concentration may be detrimental to the metabolite biosynthesis, although cell growth was not inhibited. In ganoderic acid biosyntesis by *G. lucidum*, the inhibitory effect of osmotic pressure caused by relatively high initial sugar concentration on metabolite biosynthesis was claimed. The performance of a bioreactor is greatly influenced by morphological character of the cells mediated by shearing effect.

The influence of carbon sources for biomass and EPS production of *Grifola frondosa* demonstrated that with the high concentration of carbon, increase the production of EPS. In contrast, the dry cell weight showed no substantial increase when the concentration of carbon source increased. The high concentration of carbon sources can be utilized to improve the production of EPS; however, good growth may not be a determining factor for high production of EPS in *G. frondosa* culture. A similar conclusion has been drawn from culture studies of various edible mushrooms such as *Antrodia cinnamomea* by Shih *et al*. 2006, *Pleurotus pulmonarius* by Nour *et al*. (2004), *Phellinus linteus* by Kim *et al*. (2002) and *G. lucidum* by Sone *et al*. (1985) (Table 2).

G. applanatum demonstrated that the more concentration of carbohydrate in the media, result in high production of EPS (Lee *et al.*, 2007). In contrast, EPS production of *Tremella fuciformes* was not dependent on initial glucose concentration (Cho *et al.*, 2006).

3.3 Nitrogen source

Organic and inorganic nitrogen sources were investigate in order to compare mycelia growth and polysaccharides production.

Pockhrel and Ohga (2007) tested eight different nitrogen sources individually employed. Among them, yeast extract yield the highest mycelia growth with 7.03 g/L, as well as EPS and IPS with 1.76 g/L and 325 mg/g dry mycelia growth. A similar result was reported in *Cordyceps jiangxiensis* and *Lentinus subnudus* on mycelial growth. The stimulatory effect of yeast extract is due to its protein, amino acid, and vitamin content. Various concentrations of yeast extract were applied to identify suitable concentration for mycelial growth and polysaccharide production. The yeast extract of 1% figured significantly in the stimulation of the maximum mycelial growth and inner polysaccharide production, whereas EPS production was further improved by increasing concentration of yeast extract had a significant effect on EPS production (2.46 g/L in 2%).

Mycelial growth appeared to be stimulated by all organic sources. Comparative to organic nitrogen sources, the inorganic nitrogen sources were not efficient for mycelial growth, whereas polysaccharide production improved greatly. In general, good mycelial growth does not seem to be a determining factor for high production of polysaccharide; however, this finding may not be applicable in the case of glucose concentration. Similar result was claimed in *Antrodia cinnamomea* and *Pleurotus pulmonaris* and *Pleurotus tuber-regium* (Pockhrel and Ohga, 2007; Wu *et al.*, 2003).

Jung *et al*. (1997) noted that inorganic nitrogen sources gave rise to relatively lower mycelial biomass and EPS productions of *Phellinus igniarius*. Phenomenon also observed in *Grifola frondosa* with organic nitrogen sources, and in other mushroom cultivation (Shih *et al.*, 2008). A similar result was obtained for *Agrocybe cylindracea* and *T. fuciformes* (Cho *et al.*, 2006; Kim *et al.*, 2005).

For *Auricularia auricular*, the maximum EPS production was achieved when soybean powder was used. Higher mycelial biomass and EPS concentration were observed when organic nitrogen sources were used in comparison with inorganic nitrogen sources (Table 2) (Wu, Ding and Zhang, 2006).

Mao and Zhong (2006) demonstrated that types and concentrations of nitrogen sources strongly influenced cell growth and polysaccharides production and combined use of yeast extract and corn steep liquor enhanced the accumulation of extracellular polysaccharide of *Tremella mesenterica*.

Some compounds (monosaccharides, amino acids, vitamins and others) are absorbed by cell without alterations for distinct mechanisms. However, higher molecules, such as polysaccharides and peptides have been hydrolysated to absorption of these compounds. The enzymatic activity is characteristic of each species. The growth of fungus has relation with constitutive enzymes that are present, or the substrate can induce the enzymatic activity, or the fungi can produce adaptable enzymes in the presence of uncommon substrate.

Table 2: Production of EPS by different carbon and protein sources on submerged fermentation

Species of Mushrooms	EPS (g/L)	Nitrogen Sources	Carbon Sources	References
Agaricus brasiliensis	0.32	Yeast extract	Sucrose	Fan *et al*., 2007
Agaricus brasiliensis	1.56	Peptone	Sucrose	Zou, 2006
Agrocybe cylindracea	1.24	Meat peptone + yeast extract	Glucose	Kim *et al*., 2005
Armillaria luteo-virens	5.4	Yeast extract	Glucose	Jiao *et al*., 2008
Auricula Auricularia	8.7	Soybean	Glucose	Wu, Ding and Zhang, 2006
Fomes fomenarius	3.64	Yeast extract	Glucose	Chen *et al*., 2008
Grifola frondosa	1.35	Yeast extract	Maltose	Lee *et al*., 2008
Grifola frondosa	1.32	Peptone	Glucose	Cui *et al*., 2006
Grifola umbellata	1.12	Skim Milk	Glucose	Huang and Liu, 2007
Lyophyllum decastes	1.65	Yeast extract	Glucose	Pokhrel and Ohga, 2007
Phellinus linteus	2.2	Peptone	Glucose	Zou, Sun, Guo, 2006
Phellinus baumii	2.36	Peptone	Glucose	Luo *et al*., 2009
Sarcodum aspratus	2.68	Yeast extract	Glucose	Joo *et al*., 2004
Tremella fuciformes	1.45	Tryptone	Fructose	Cho *et al*., 2006
Tuber malanosporum	2.2	Peptone + yeast extract	Sucrose	Liu *et al*., 2009

3.4 Mineral source effect

The effect of mineral sources on mycelial growth and EPS production have usually been recognized as favorable for mycelial growth and EPS production in liquid cultures of several basidiomycetes (Kim *et al*., 2005). Chardonnet *et al*., (1999) investigated that external Ca^{+2} can play an indirect role in fungal growth by altering internal Ca^{+2} that controls the cytoplasmic Ca^{+2} gradient, and activity of fungal enzymes involved in cell wall expansion. Direct effect of Ca^{+2} on the fungal cell wall may also be

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a significant factor in cell membrane permeability interactions. In contrast, Papagiani (2004) suggested that Ca^{+2} accumulations seemed to inhibit the biosynthesis of fungal biopolymers, possibly through an effect on enzymes such as ß-glucan-synthases. For higher CaCl concentrations, the calcium ion content of the cell wall increased, resulting in reduced protein and sugar contents. The effects of these agents appear to be mediated by tip-high gradient of cytoplasmic free Ca^{+2}, which is obligatorily present and involved in active growth. Mg^{+2} are essential to all fungi. It's a cofactor in enzymatic reactions, stabilizes the plasma membrane, and its uptake is ATP dependent. The positive action of Ca^{+2} and Mg^{+2} on mycelial growth and EPS production was obvious in the present submerged cultures.

Jonathan and Fasidi, 2001 studied the influence of macro-elements and micro-elements about mushroom growth, *Psathyrrella atroubonata*. Data suggest that calcium, magnesium, potassium and sodium were most utilizable by mushroom, followed in order. Calcium and Magnesium are important in metabolism. The absence of cobalt and manganese (microelements) improve biomass growth can indicate toxicity to mushroom cells.

3.5 pH Effect

The pH of medium is important factor that contributed mycelial growth and polysaccharide production. The medium pH may affect cell membrane function, cell morphology and structure, the uptake of various nutrients, and product biosynthesis.

Many investigators claimed that the different morphology of fungi mycelia under a different initial pH value was the critical factor in biomass accumulation and metabolite formation (Pokhrel and Ohga, 2007; Kim *et al.*, 2005).

In general, the process of submerged fermentation of mushroom, the culture pH showed a sharp decrease to 3.2 during 4 days of cultivation. After that, the pH remained relatively constant for about 1 week. At the end fermentation (10 - 14 days), when the residual glucose was almost exhausted, the pH values quickly increased to 7.0. The pH increase towards the end of the culture was considered to be related with debris cells, resulting of autolysis process (Tang, 2008).

Shih *et al*. (2008) showed that the optimal initial pH for both mycelial biomass and EPS production for *Grifola frondosa* in the submerged culture was pH 5.5. These are consistent with the fact that many kinds have acidic pH optima during submerged cultures. The higher biomass production associate with the lower pH in the medium and usually attributed to the production of organics acids.

Antrodia camphorate demonstrated that the culture pH in submerged culture significantly influenced the antioxidant and free radical scavenging activities of methanolics extracts of filtrates and methanolics extracts of mycelia in submerged culture. The optimal culture pH was 5.0 in terms of antioxidant activities, which had been successfully correlated with the total phenolics, polysaccharide contents, and protein/polysaccharide ratio (Shu and Lung, 2008).

The optimum nutritional and environmental conditions for EPS production in shake flasks by *Auricularia auricula* was pH 5.4 initial. The results demonstrated that the EPS production was strongly associated with mycelial growth. The maximum concentration of EPS (4.5 g/L) was achieved after 96 hours. The decrease rate of pH in the culture broth was slow at early period (0 - 48 hours), and then became fast during 48-96 hours (pH dropped to 3.96). At the final phase, pH remained unchanged at 3.83. The favorite pH level for EPS production by *A. auricula* was higher than 4.0 (Wu, Ding and Zhang, 2006).

Cho *et al*. (2006) and Hwang *et al*. (2005) demonstrate that *Tremella fuciformis* and *Phellinus gilvus*, respectively, have optimum growth at an alkaline pH (8 and 9).

3.6 Temperature effect

Mushrooms have relatively low temperature optima (20 – 25°C) in their submerged cultures (Kim *et al*., 2002; Bae *et al*., 2000). Lee *et al*. (2007) demonstrated the highest amount of EPS was obtained in the culture grown at 25°C for *Ganoderma applanatum*. The cell biomass and intrapolysaccharide content in the cell were higher in cultures grown at 10°C and tended to decrease as the culture temperature increase. The results suggest that the mycelia accumulate polysaccharides in the cell at low temperature. These data indicate that the optimal temperature for production of

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endopolysaccharide is different from that of EPS for *Ganoderma applanatum*. Similar results for EPS were reported from submerged culture of *Agrocybe cylindracea* and *Grifola frondosa*.

The temperature optimum of *Tremella fuciformis* was 28°C to growth as seem in the results obtained from other liquid cultures of kinds of mushrooms (Cho *et al.*, 2006).

3.7 Aeration rate effect

Aeration is very important for stimulate oxidative reactions, energy generation, maintenance cellular and influence the formation and accumulation of bioactive metabolites in submerged fermentation.

Mycelial biomass and polysaccharides production from fermentation of mushroom are mainly affected by the type of bioreactor and its aeration or agitation rate (Lee *et al.*, 2007).

T. fuciformis targeted the maximum cell mass and EPS production (2.0 g/L^{-1}) were achieved at 200 rpm. High levels of cell mass and EPS production were observed at highest aeration rate of 2 vvm. Tests with aeration rate of 2 vvm, the dissolved oxygen level increased rapidly as the growth entered a stationary phase, thereafter high dissolved oxygen levels (over 50%) were maintained towards the end of fermentation. These results suggest that maintenance of high dissolved oxygen levels is important for both cell growth and EPS formation in *T. fuciformis* fermentation. Several investigators have reported similar results during secondary metabolite production in batch fermentations (Cho *et al.*, 2006).

Kurbanoglu *et al.*, 2004 showed the highest biomass yield was obtained from 150 rpm for 8 days. The biomass yield at 200 rpm conditions was lower. The answer for this result may be explained from the detrimental effect of increased oxygen and stress on the mycelium.

3.8 Inoculum density

Inoculum density (or inoculum size) is important culture factor for submerged fermentation of many mushrooms. In all cases, the lag phase was observed within the range of inoculation density as investigated. Fang and Zhong (2002) reported that a small inoculum size led to a low final cell density during the submerged fermentation of *Ganoderma lucidum* in shake flasks, an inoculation size of 170 mg of dry weight (DW/L) was necessary for fermentation production, and maximal cell concentration of 15.7 g DW/L was obtained at inoculation density of 330 mg DW/L (Tang *et al.*, 2008).

Tang and Zhong (2002) reported that the inoculation density significantly affected EPS production. The relatively high EPS production was obtained at large inoculum size in the *G. lucidum* fermentation and the maximal *Ganoderma* EPS production titer of 0.88 g/L was obtained at highest inoculation density of 670 mg/L on day 8.

Hence a mycelial inoculum with a volume ratio of 2 mL per 100 mL of liquid medium seemed to be optimum for growth of *Pleurotus tuber-regium*. The reason why a larger volume of inoculum did not increase mycelial yield was not clear but it is possible that the yield was limited by the amount of nutrients available in the medium (Wu *et al.*, 2003).

During the submerged fermentation of medicinal mushroom *Cordyceps jiangxiensis*, both mycelial biomass and intracellular polysaccharides (IPS) production were both near optimal values at inoculum sizes of 4-6% and declined rapidly outside this range (Xiao *et al.*, 2006). The process submerged fermentation of mushroom *Agaricus blazei*, the optimum inoculum size for both mycelial growth and EPS production was identified to be 10% (v/v) in shake flask cultures (Han *et al.*, 2004). These works indicate that the control of inoculation density was very important to both the cell growth and polysaccharides production during the submerged fermentation of mushroom.

3.9 Time of fermentation effect

The lag phase is a period during which an increase in cell size or protein content is occurring in the growth of fungal culture (Wu *et al*., 2003).

In general, the harvest time of the mycelium, in complex carbohydrate medium should not extend beyond 20 days after the inoculation in order to avoid fungal cell lysis. Similarly, a decrease in yield in this fungus on day may have been caused by fungal cell lysis. Comparing before and after 10-day cultivations, both EPS and IPS were much higher in earlier (on day 5) than after 10-day cultivations, yet mycelial yield was enhanced in the later rather than the earlier. This result showed that the activity of polysaccharide production was rather short – phased, even though mycelial growth continues for a longer duration. This result implies that the highest mycelia yield and polysaccharide production, changes with cultivation time periods (Pokhrel, and Ohga 2007).

Table 3: Environmental parameters of submerged fermentation of mushroom for EPS production

Mushrooms	pH	Temp. (^{o}C)	Time (days)	Agitation (rpm)	References
Agaricus bisporus	6.0	26	8	150	Kurbanoglu *et al*., 2004
Agaricus brasiliensis	6.1	30	7	150	Fan *et al*., 2007
Agaricus brasiliensis	6.0	25	6	180	Zou, 2006
Agrocybe cylindracea	6.0	25	10	150	Kim *et al*., 2005
Armillaria luteo-virens	5.0/5.5	23	5	100	Jiao *et al*., 2008
Auricuaria auricula	5.5	28	4	150	Wu, Ding and Zhang, 2006
Collybia maculata	5.5	20	5	150	Lim and Yun, 2006
Fomes fomentarius	5.0/6.0	25	7	150	Chen *et al*., 2008
Ganoderma applanatum	4.5	25	12	100	Lee *et al*., 2007
Grifola frondosa	5.5	25	10	80	Cui *et al*., 2006
Lyophyllum decastes	7.0/8.0	25	10	125	Pokhrel and Ohga, 2007
Tremella fuciformis	8.0/9.0	28	4	200	Cho *et al*., 2006

The optimum production of EPS from mushroom had pH since 4.5 until 9.0, for *G. applanatum* and *T. fuciformes*, respectively. The temperature for major mushroom was around 25-28°C. Flasks or bioreactor agitation that promote mixture of nutrients and permit the aeration of system fermentation showed agitation frequencies of 80-200 rpm. The period of fermentation for maximum production of EPS is very important, mainly for industrial aspects. Reduce time of fermentation with higher EPS production is objective to be reached.

4 Conclusion

The consumption of mushroom or isolated bioactive compounds is beneficial to health, such as heart diseases, diabetes, tonic, circulatory system, immunomodulatory effects and anti-cancer. Biomolecules of mushroom which are found in nature can be produced by submerged fermentation. Exopolysaccharides and polysaccharide-protein complexes are recognized such as bioactive molecules with potential for treating several diseases. The ß-glucans are constituents of fungal cell wall, but may be excreted into the medium in fermentation. This review showed some possibilities of submerged fermented of mushrooms for EPS production. Each species of Macromycetes have optimum conditions to produce bioactive compounds. Studies of submerged fermentations of mushrooms have showed good perspectives for industrial application, with advantages such as: reduced space, quickly and safe. There are many possibilities, alternative sources of carbon and nitrogen, micronutrients, growth factors and new mushrooms species for investigate.

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Chapter III

ANTIOXIDANT ACTIVITIES OF EDIBLE MUSHROOM COMPOUNDS PRODUCED BY SUBMERGED CO-CULTURE

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Abstract

The antioxidant potential of mushrooms such as *Agaricus brasiliensis*, *Cordyceps sinensis*, *Ganoderma lucidum* and *Grifola frondosa* cultivated separately and in combination by submerged fermentation was investigated. The co-culture these species resulted in six combinations: *A. brasiliensis* + *C. sinensis*, *A. brasiliensis* + *G. lucidum*, *A. brasiliensis* + *G. frondosa*, *C. sinensis* + *G. lucidum*, *C. sinensis* + *G. frondosa*, *G. lucidum* + *G. frondosa*. The submerged cultivation of mushrooms resulted in three bio-compounds: ethanolic biomass extracts (EBE), fermented broth (BF) and exopolysaccharide (EPS). These compounds were evaluated for their antioxidant activity in different mechanisms: free radical scavenging capacity, inhibition of lipid peroxidation, reducing power, anion superoxide scavenging activity and total phenol compounds. DPPH capacity of biomass from *G. lucidum* + *G. frondosa* showed radical affect 79.742% ± 1.72 and isolate culture of *G. lucidum* 79.097% ± 3.629. These results are similar. The best result for the inhibition of lipid peroxidation ß-carotene + linoleic acid systems was obtained with the ethanolic biomass extracts of *A. brasiliensis* + *C. sinensis* was 28.63% ± 5.62 was the better results for inhibition of lipid peroxidation ß-carotene + linoleic acid systems, but it is lower compared to controls. Reducing power activities showed results close to controls in most samples. *A. brasiliensis* + *G. lucidum* presented 127.58% ± 2.4 for superoxide anion and 168.84 ± 10.29 µg/mL Gallic Acid equivalent for quantify phenol compounds. The synergic submerged fermentation of mushrooms is alternative for obtaining natural compounds with antioxidant activity and similar effect to traditional substances.

Key Words: Macromycetes, free radicals, antioxidant properties, submerged fermentation

1 Introduction

During the reduction of molecular oxygen, reactive oxygen species are formed and there is a continuous requirement for inactivation of these free radicals. The imbalance between oxidant and antioxidant molecules results in free radicals formation, which is known as oxidative stress. Free radicals can be generated in the cytoplasm, in mitochondria or the cell membrane. Damage induced by free radicals can affect many biological molecules, including lipids, proteins, carbohydrates and vitamins (Bianchi and Antunes, 1999).

Polyunsaturated fatty acids are abundant in cells and are susceptible to oxidation due to the presence of methylene group between double bonds. It is estimated that approximately 60 molecules of linoleic acid and 200 of arachidonic acid (most abundant fatty acid in our cells) are consumed by oxidant compounds that react with cellular lipidic bilayer. The oxidative reaction initiates an autocatalytic cascade, generating numerous genotoxic substances, damaging lipids, forming hydroperoxides, and producing secondary metabolites (Loureiro, Mascio and Medeiros, 2002).

Oxidative stress can affect biological molecules and decrease the antioxidant capacity of the system. It can be involved in the pathogenesis of diseases, such as cancer, rheumatoid arthritis, cardiovascular diseases, cirrhosis and arteriosclerosis as well as in degenerative processes associated with ageing (Shu and Lung, 2008; Bianchi and Antunes, 1999).

Antioxidants can inhibit oxidation of various substrates, from simple molecules to polymers and complex biosystems, by two mechanisms: the first one comprises inhibiting the formation of free radicals that enable the initiation step, the second includes the elimination of important radicals in the propagation step, such as alkoxyl and peroxyl, by donating hydrogen atoms to these molecules, therefore interrupting the chain reaction

All organisms are equipped with several defense systems against free radical damage and produce antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase as defense tools against free radicals damages. Oxidation processes can be avoided by change in environmental conditions or the use of

antioxidants to prevent or reduce oxidative reactions. The majority of human diseases can have their risks decreased with diets rich in antioxidants, containing supplements such as α-tocopherol, ascorbic acid, flavonoid, carotenoids, polyphenol compounds and glutathione (Barros *et al.*, 2007; Ferreira and Matsubara, 1997). Natural antioxidants are being extensively studied for their capacity to protect organisms and cells from damage brought on by oxidative stress (Kil *et al.*, 2009; Barros *et al.*, 2008).

Mushroom is known to possess important roles in human diet and disease prevention, on account of their bio-compounds which present biological properties like antioxidant activities. Not only can mushroom products be used as food supplements, but also as pharmaceutical components.

It have been shown that mushroom species possess antioxidant activity *in vitro* systems and have the ability to accumulate a variety of secondary metabolites, including organic acids, alkaloids, polysaccharides, triterpenoids, steroids phenolics compounds, polyketides, terpenes and steroids (Turkuglu *et al.*, 2007; Shu and Lung, 2008; Siddhuraju and Becker, 2007).

Submerged fermentation of mushrooms is an alternative for obtaining active metabolites. Fermentation process parameters have significant influence in the physiology of the microorganism, and consequently it implicates in the quality and content of physiologically active substances. In fact, aspects as strain, culture, temperature, pH are important for the suitable growth of the mushroom. The submerged fermentation permits a better control of culture conditions and has become essential to meet the increasing demands in the international markets (Fan *et al.*, 2005; Wu *et al.*, 2006).

Therefore, this work aimed the evaluation of antioxidant properties of ethanolic biomass extract, fermented broth and EPS from mushrooms cultivated separately and in co-cultivation by submerged fermentation. The experiments were carried out with the following Macromycetes species: *Agaricus brasiliensis, Cordyceps sinensis, Ganoderma lucidum and Grifola frondosa*.

2 Materials and Methods

2.1 Mushroom Strains

Strains of *Agaricus brasiliensis, Cordyceps sinensis, Ganoderma lucidum and Grifola frondosa,* were acquired from the collection bank of Biotechnology and Bioprocess Laboratory of Federal University of Paraná (Brazil). They were cultivated on Potato Dextrose-Agar (PDA) plate at 25°C for 7 days. All experiments were carried out using the 7-day-old mycelium to inoculate the flask medium.

2.2 Submerged Fermentation of Mushrooms

2.2.1 Pre-inoculum

Mushrooms were grown on PDA medium in Petri dish, and then transferred into the seed culture medium by pushing out 5 mm of the agar plate culture with a self-designer cutter. Erlenmeyer flasks of 250 mL containing 100 mL of basal medium were inoculated with mycelium and incubated at 28°C for 7 days, and then used for pre-inoculum. After fermentation, the biomass were filtrated, macerated under sterile conditions and resuspended in 100 mL of sterile culture medium.

2.2.2 Co-cultivation inoculum

Submerged fermentations were conducted in 500 mL shake flasks containing 250 mL of liquid medium, which were incubated at 28°C on a rotary shaker (120 rpm) for 7 days. The fermentation medium was inoculated at 8% (v/v) of the above seed culture medium and kept at 28°C ± 2 and 120 rpm for a period 6-7 days. For submerged fermentation with two species, the inoculum was 4% (v/v) for each strain in the same conditions. The culture medium consisted of the following components (g/L): glucose, 20; yeast extract, 3; KH_2PO_4, 0.6; $MgSO_4$, 0.3; pH 6.1, according Fan *et al*. (2005) (modified).

Mushrooms were incubated at 28°C $\pm$ 2, 120 rpm in shaker. The combinations among mushrooms species for co-cultivation are presented in table 1:

Table 1: Macromycetes cultivated by synergic submerged fermentation

Mushrooms Species	
Agaricus brasiliensis	*Ganoderma lucidum*
Grifola frondosa	*Cordyceps sinensis*
Co-cultivation of Mushrooms	
1-A. brasiliensis + C. sinensis (Ab+Cs)	*4- C. sinensis + G. lucidum (Cs+Gl)*
2- A. brasiliensis + G. lucidum (Ab+Gl)	*5- C. sinensis + G. frondosa (Cs+Gf)*
3- A. brasiliensis + G. frondosa (Ab+Gf)	*6- G. lucidum + G. frondosa (Gl+Gf)*

2.2.3 Compounds extraction from mushrooms

After 7 days of submerged fermentation mushroom, each sample of co-cultivation and controls were filtered and obtain biomass, fermented broth and EPS precipitation. These bio-compounds from mushroom had their antioxidant effect evaluated: free radical scavenging capacity, inhibition of lipid peroxidation, reducing power, anion superoxide scavenging activity and total phenol compounds.

a) Ethanolic biomass extracts (EBE): to prepare the ethanolic extracts of biomass, it was added to the filtered mycelia 100 mL of absolute ethanol and it was kept under agitation for 12 hours in shaker at 120 rpm. The ethanolic biomass extracts were filtrated and evaporated in rotary-evaporator at 50°C. To the remaining fraction on it was added 10mL of ultra-pure water and the solution was maintained at -20°C in freezer.

b) Fermented broth (FB): after filtration processes, the fermented broths had an aliquot of 20 mL collected for antioxidants assays.

c) Exopolysaccharide (EPS): Aliquots of 100 mL of fermented broths were concentrated under reduced pressure and added with volumes of ethanol. The solutions were then maintained overnight at 4°C. The precipitates were separated by centrifugation at 4000 rpm for 20 minutes/10°C and dissolved in ultra-pure water (Figure 1).

d) Controls: mushrooms species were cultivated isolated in submerged fermentation and submitted the same process.

Submerged Fermentation

Controls	Co-Cultivation
Agaricus brasiliensis	*A. brasiliensis + C. sinensis*
Cordyceps sinensis	*A. brasiliensis + G. lucidum*
Ganoderma lucidum	*A. brasiliensis + G. frondosa*
Grifola frondosa	*C. sinensis + G. lucidum*
	C. sinensis + G. frondosa
	G. lucidum + G. frondosa

Filtration

Biomass — Fermented Broth — **Fermented Broth (1)**

Fermented Broth (EPS)

Ethanolic Extraction (12h/120 rpm)

Filtration

Rotaevaporation at 50°C

Rotaevaporation at 50°C

Ethanolic precipitation (4°C)

Water Solubilization

Biomass extracts (3)

EPS (2)

Figure 1: Scheme of sample of ethanolic biomass extraction (EBE), fermented broth (FB) and exopolysaccharide (EPS) from Macromycetes synergically cultivated. Biocompounds tested were indicated as (1), (2) and (3).

2.3 Antioxidant Activity

2.3.1 Scavenging ability on 1,1 Diphenyl-2- Picrylhydrazyl radical (DPPH)

Scavenging activity of the free radicals by bio-compounds of mushrooms was measured in terms of hydrogen atom donating, using the stable radical DPPH method. Aliquots of 1 mL of the bio-compounds of mushrooms were added to 4 mL of 0,004% methanol solution of DPPH. After 30 minutes incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical by DPPH in percentage (%) was calculated in following way (Eq 1):

$$\%= \left\{ (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}) \text{ x } 100 \right\} \qquad \text{Eq. (1)}$$

Where A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance of the test compound. The values of inhibition were calculated according the controls acid ascorbic, buthylated hydroxyanisole (BHA) and α-tocopherol (100 µg/mL) and equation (1). All tests were carried out in triplicate (Turkoglu *et al.*, 2007).

2.3.2 ß-Carotene-linolenic acid assay

The antioxidant activity of samples was evaluated by ß-carotene bleaching method describe by Turkoglu *et al.*, (2007). Firstly, ß-carotene (0.5 mg) was dissolved in a solution of 1 mL of chloroform (HPLC grade), 25 µL of linoleic acid and 200 mg of Tween 40 it was 100 mL. After evaporation of chloroform, 100mL of distilled water satured with oxygen, until form an emulsion (ß-carotene-linoleic acid emulsion). Aliquots of 4 mL of emulsion were dispensed into test tubes with 0.2 mL of the samples and incubated for 2 hours at 50°C temperature. The positive controls BHA and α-tocopherol and blank control followed the same procedure. All tests were carried out in triplicate. Absorbances of the reaction were measured at 490 nm and compared with controls and calculated by formula given below (Eq. 2).

$$(\%) \left\{ \left[A_{control} - A_{sample}/A_{control} \right] \text{x } 100 \right\} \qquad \text{Eq. (2)}$$

2.3.3 Reducing power activity

    Reducing power of samples was determined by the method of Oyaizu (1986) apud Shu and Lung (2008). Aliquots of 2.5 mL of the samples were added to sodium phosphate buffer (2.5 mL, 200 mM, pH 6.6) and potassium ferricyanide (1%, 2.5 mL) and incubated at 50°C for 20 minutes. After incubation was added trichloroacetic acid 10% (w/v) (2.5 mL) was added and centrifuged at 650 rpm for 10 minutes. The upper layer (5.0 mL) was mixed with deionized water and ferric chloride 0.1%, (1.0 mL). Absorbance was measured at 700nm, and absorbance the higher the absorbance, the better its reducing power. Tests were expressed by mean of triplicate measurements.

2.3.4 Superoxide Anion scavenging activity

    Anion superoxide scavenging activity of samples was measured according to Shu and Lung (2008). The reaction contained 1 mL of each of the following solutions prepared in 0.1 M phosphate buffer at pH 7.4, 150 μM nitroblue tetrazolium (NBT), 60 μM phenazine methosulphate and 468 μM NADH the same reaction contained 1 mL of the samples and the same volume of the following solutions. The reaction took place at ambient temperature for 5 minutes and absorbance was measured at 560 nm, against a blank in a spectrophotometer. Tests were expressed by the means of triplicate measurements. The scavenging activity on superoxide anion radicals was expressed (%), according to Eq. (3):

$$\left\{ \frac{\left[\textit{Absorbance at 560 nm in the presence of sample} \right]}{\left(\textit{Absorbance at 560 nm in the absence of sample} \right)} \right\} \text{ x 100} \qquad \text{Eq. (3)}$$

2.3.5 Determination of total phenolics compounds

The content of total phenolics compounds was determined by the spectrophotometric method of Folin-Ciocalteau using gallic acid as standard. The Folin Ciocalteau is a solution of complex polymeric ions formed by phosphomolybdic and phosphotungstic heteropolyacids. This reagent oxidizes the phenolate, reducing the acid to a blue complex. An aliquot of 0.5 mL of bio-compounds (EBE, FB and EPS) was transferred to a tube and it was added 2.5 mL of Folin Ciocalteau (Sigma Aldrich Chemical Co.) diluted 1:10 in distilled water. The reaction tube was placed for 3 to 8 minutes in dark and it was added 2 mL of sodium carbonate 4%. The tubes rested for 2 hours in the dark at room temperature and, subsequently, had their absorbance read at 740 nm. A reagent blank was carried out under the same conditions. The calibration curve was constructed with 100, 80, 60, 40, 20, 10 $\mu g.mL^{-1}$ of gallic acid and the results expressed in μg GAE.g^{-1} (Gallic Acid Equivalent). The reading was performed at 740 nm and calculated by Eq. (4) (Minussi *et al.*, 2003; Kroyer and Hegedus, 2001; Singleton *et al.*, 1965).

$$\left\{ \begin{array}{c} Absorbance= 0.0275 \mu g\ pyrocatecol + 0.0209 \\ R^2= 0.9808 \end{array} \right\} \quad \text{Eq. (4)}$$

3 Results and Discussion

3.1 Scavenging ability on 1,1 diphenyl-2- picrylhydrazyl radical (DPPH)

Ethanolic biomass extracts (EBE), fermented broth (FB) and exopolysaccharides (EPS) exhibited varying degrees of scavenging capacities. The best scavenging capacity was achieved by ethanolic biomass extracts of co-cultivation with *G. lucidum* + *G. frondosa* which showed radical effect of 79.74% ± 1.72, followed by the isolate culture of *G. lucidum* with 79.09% ± 3.62 (P >0,05) and *C. sinensis* + *G. frondosa*, that showed 70.34 ± 2.33. Scavenging effects of fermented broth of *G. frondosa* presented 77.80 ± 2.23. Additional, co-cultivation of *A. brasiliensis* + *G. lucidum* and *A. brasiliensis* + *G. frondosa* obtained 68.69 ± 1.88 and 68.60 ± 0.89, respectively to scavenging ability. These results were higher than α-tocopherol, a natural compound used as control (66.25 ± 2.95) (Table 2). Exopolysaccharides showed results ranging from 38.62 ± 1.95 (*A. brasiliensis* + *G. frondosa*) to 49.44 ± 1.53 (*G. frondosa*) for scavenging activity.

Table 2: Scavenging capacity of the extracts of Macromycetes synergic cultivate by submerged fermentation on 1,1-diphenyl-2-picrylhydrazyl – DPPH

Mushrooms cultivated by submerged fermentation (%)			
A. brasiliensis (*Ab*)	*C. sinensis* (*Cs*)	*G. lucidum* (*Gl*)	*G. frondosa* (*Gf*)
EBE 52.94 ± 2.34	46.36 ± 1.72	79.09 ± 3.62	67.72 ± 1.17
FB 59.89 ± 2.96	61.60 ± 4.20	50.78 ± 1.43	77.80 ± 2.23
EPS 40.42 ± 1.15	41.94 ± 2.78	42.95 ± 1.49	49.44 ± 1.53

Mushrooms co-cultivation by submerged fermentation					
Ab + Cs	*Ab + Gl*	*Ab + Gf*	*Cs + Gl*	*Cs + Gf*	*Gl + Gf*
EBE 49.53 ± 5.9	69.75 ±1.8	46.31 ± 1.5	47.69 ± 1.6	70.34 ± 2.2	79.74 ± 1.7
BF 58.37 ± 3.7	68.96 ±1.9	68.60 ± 0.9	63.07 ± 0.5	52.16 ± 4.3	59.94 ± 0.5
EPS 46.132 ± 2.3	43.96 ±4.1	38.62 ± 1.9	40.05 ± 3.1	48.71 ± 1.6	47.14 ± 1,9

Controls			
BHA (100 µg/mL): 90.14 ± 1.97		α-Tocopherol (100 µg/mL): 66.25 ± 2.95	

*Ethanolic Biomass Extract (EBE); Fermented Broth (FB) and Exopolysaccharide (EPS).

DPPH is a stable free radical and accepts an electron or hydrogen radical. This compound shows characteristic absorption at 517 nm (purple). In the DPPH test, the antioxidants were able to reduce the stable DPPH radical and the decreasing in absorbance (yellow), because of the reaction between antioxidant molecules and the radical, which results in the scavenging of the radical by hydrogen donation (Chattopadhyay *et al.*, 2009; Elmastas *et al.*, 2007).

Liu *et al.*, (2010) obtained two low-molecular-weight exopolysaccharides purified from a crude *G. lucidum.* The results indicated that one EPS fraction was a glucan with an average molecular weight of 5.2 kDa, while the other had a molecular weight of 15.4 kDa. Antioxidant results showed that both EPS exhibited antioxidant activities. Low-molecular-weight polysaccharide seems to play an important role in the exploration of natural antioxidants.

Fruiting bodies of *A. brasiliensis* showed scavenging effect around 90% at concentration of 6 mg/mL, but this value was considerably lower than that reported by Huang, Huang, Chen, and Mau (1999), who found a high scavenging ability of 97.1% at 2.5 mg/mL for the methanolic extract from *Agaricus blazei* (Soares *et al.,* 2009). Elmastas *et al.* (2007) analyzed extracts of mushroom fruiting bodies having effect on scavenging free radical and *A. bisporus* showed 82.8%. Sarikurkcu *et al.*, (2008) and Ribeiro *et al.*, (2008) showed radical scavenging effect of *Boletus edulis* was 94.66% and 94.9% respectively. Ethanolic extracts showed scavenging abilities on DPPH radicals of 94.9, 89.2, 88.8 (%) for *A. blazei*, *Agrocybe cylindracea* and *Boletus edulis*, respectively (Tsai, Tsai and Mau, 2007).

According with studies mushroom has a noticeable effect on scavenging free radical. Ethanolic and methanolic extraction method of fruit bodies showed more effective than submerged fermentations extracts. However, conditions of submerged fermentation and extraction methods can be optimized for produce antioxidant compounds.

3.2 ß-Carotene-linolenic acid assay

Inhibitory activity of lipid peroxidation from bio-compounds of mushroom was lowest, compared with controls, BHA (99.14 ± 0.72) and α-tocopherol (97.69 ± 3.03).

The better results was of ethanolic biomass extract (EBE) of *A. brasiliensis + C. sinensis* with 28.63% ± 5.62, while species isolates was 13.95% ± 1,05 and 15.32% ± 0.84, for *A. brasiliensis* and *C. sinensis*, respectively. Fermented broth (FB) of *G. frondosa* showed 22.91% ± 0.72 and exopolysaccharide (EPS) of *G. lucidum* was 18.95% ± 0.50 antioxidant activities (Table 3).

Table 3: Antioxidant activity (%) of the methanolic extracts of mushroom by ß-carotene-linoleic acid method

Compounds*	Mushrooms cultivated by submerged fermentation			
	A. brasiliensis (*Ab*)	*C. sinensis* (*Cs*)	*G. lucidum* (*Gl*)	*G. frondosa* (*G.f*)
EBE	13.95 ± 1.05	15.32 ± 0.84	13.55 ± 0.13	6.53 ± 0.27
FB	12.34 ± 0.50	2.90 ± 0.50	1.53 ± 0.13	22.91 ± 0.72
EPS	11.21 ± 0.50	17.42 ± 0.73	18.95 ± 0.50	16.78 ± 0.50

Compounds*	Mushrooms cultivated synergically by submerged fermentation					
	Ab + *Cs*	*Ab* + *Gl*	*Ab* + *Gf*	*Cs* + *Gl*	*Cs* + *Gf*	*Gl* + *Gf*
EBE	28.63 ± 5.6	0.48 ± 0.03	13.31 ± 0.7	14.60 ± 0.6	15.4 ± 0.7	1.21 ± 0.27
FB	12.26 ± 0.7	12.02 ± 0.6	11.9 ± 0.73	2.3 ± 0.24	2.0 ± 0.3	11.5 ± 0.5
EPS	11.69±0.5	11.85±0.1	12.02±0.4	12.18±1.0	8.07±0.8	18.07±0.2

Controls

BHA (100 μg/mL): 99.14 ± 0.72%   α-Tocopherol (100 μg/mL): 97.69 ±3.03%

*(EBE) Ethanolic Biomass Extract; (FB) Fermented Broth and (EPS) Exopolysaccharide.

Oxidative deterioration of polyunsaturated lipids is a process caused by free radicals, which is known by lipid peroxidation. This reaction may inactivate cellular components and plays a role in oxidative stress in biological systems. Transition metal ions, such as iron and copper, are known to stimulate lipid peroxidation through various mechanisms. These metal ions may generate hydroxyl ions (OH⁻) to initiate the lipid

peroxidation process and/or propagate the chain process via decomposition of lipid hydroperoxides (Jayakumar *et al.*, 2009).

ß-carotene-linoleic acid method antioxidant evaluates the inhibition of free radicals activities generated during linoleic acid peroxidation. Peroxyl radicals are formed by a direct reaction of oxygen with alkyl radicals. The free radical linoleic acid attacks the highly unsatured ß-carotene, and the presence of antioxidant can hinder extend of ß-carotene-bleaching by neutralizing the linoleate free radical (Ferreira *et al.*, 2009; Soares *et al.*, 2009; Siddhuraju *et al.*, 2007).

Methanolic extracts of fruit bodies from *Lactarius deterrinus*, *Suillus collitinus*, *Boletus edulis*, *Xerocomus chrysenteron* showed inhibition on peroxidation in linoleic acid system were 97.94, 97.85, 97.08, 95.16 (%) respectively, at 20 mg/mL of compounds concentration (Sarikurkcu *et al.*, 2008). Soares *et al.* (2009) obtained fruit body extracts of *A. brasiliensis* with concentration of 10mg/mL inhibited 90% of peroxidation lipidic.

Jayakumar *et al.* (2009) showed at concentration of 10 mg/mL, the *Pleurotus ostreatus* extract achieved 56.20 % inhibition of lipid peroxidation and the ascorbic acid standard, 67.15 %.

Results obtained ß-carotene-linoleic acid method were different of DPPH, because the mechanism action of first method determines activity of sample or compound to protect a substrate of lipid oxidation, while the methods of inhibition of DPPH radical based it on transfer of electrons from antioxidant to oxidant (Duarte Almeida *et al*, 2006).

Biomass extract and broth fermented by submerged fermentation of mushrooms showed inhibition on transfer of electrons in antioxidant mechanism than protect a substrate of lipid oxidation.

3.3 Reducing power activity

Reducing power activity of bio-products from mushrooms cultivated by submerged fermentation in co-cultivation demonstrated results close to controls (Figures 2, 3 and 4).
The controls BHA and α-tocopherol presented 1.305 ± 0.027 and 1.382 ± 0.0825 of absorbance at 700 nm, respectively.

Biomass ethanolic extracts from *G. lucidum* (B3) obtained absorbance of 1.293 ± 0.028 and *A. brasiliensis + C. sinensis* (B5) was 1.28 ± 0.010, not showed statistical differences compared to controls. *C. sinensis* (B2) and *G. frondosa* (B4), isolated cultive, presented lowest results, 1.037 ± 0.05 and 1.10 ± 0.03, respectively. Co-cultivation of *A. brasiliensis + G. frondosa* (B7), *C. sinensis + G. lucidum* (B8), *C. sinensis + G. frondosa* (B9) and *G. lucidum + G. frondosa* (B10) obtained 1.22 ± 0.06, 1.23 ± 0.07, 1.24 ± 0,05 and 1.26 ± 0,014 for reducing power activity, not showed statistical difference (Figure 2).
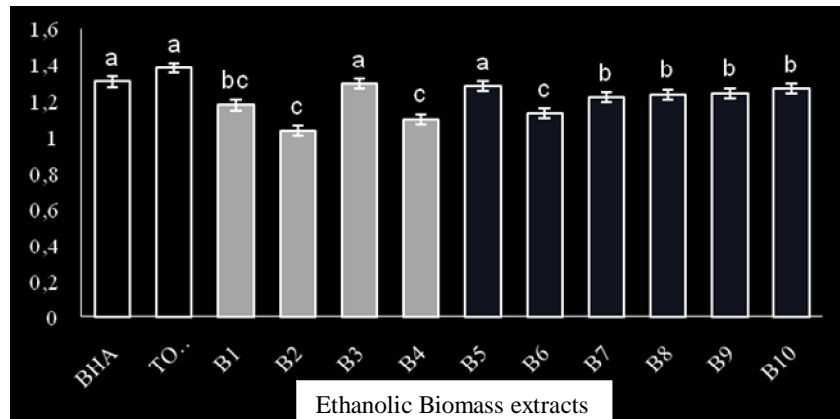
Figure 2: Reducing power of ethanolic biomass extracts from mushroom co-cultivated by submerged fermentation. Mushrooms and co-cultivations: B1- *A. brasiliensis*, B2- *C. sinensis*, B3- *G. lucidum*, B4- *G. frondosa*, B5- *A. brasiliensis + C. sinensis*, B6- *A. brasiliensis + G. lucidum*, B7- *A. brasiliensis + G. frondosa*, B8- *C. sinensis + G. lucidum*, B9- *C. sinensis + G. frondosa*, B10- *G. lucidum + G. frondosa*. Each value is expressed as means ± standard deviation (n=3). Means with different letters within a column are significantly different ($P<0.05$).

Broth fermented of co-cultivation of mushroom showed absorbance at 700 nm of 1.36 ± 0.051 and 1.33 ± 0.035 of *C. sinensis + G. frondosa* (E9) and *G. lucidum + G.frondosa* (E10), respectively (Figure 3). These results not showed statistic differences compared to control α-tocopherol (1.382 ± 0.0825). Broth fermented samples: E1- *A. brasiliensis*, E2- *C. sinensis*, E3- *G. lucidum* (isolated cultivation) and E6- *A. brasiliensis + G. lucidum*, E7- *A. brasiliensis + G. frondosa*, E8- *C. sinensis + G. lucidum* (co-cultivation) obtained data close to control BHA (1.305 ± 0.027).
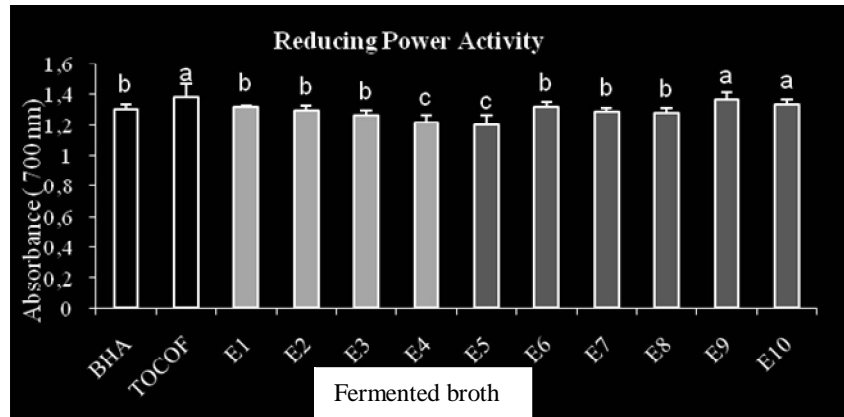
Figure 3: Reducing power of fermented broth from mushroom co-cultivation by submerged fermentation. Fermented broth from mushrooms produced by submerged fermented: E1- *A. brasiliensis*, E2- *C. sinensis*, E3- *G. lucidum*, E4- *G. frondosa*, E5- *A. brasiliensis + C. sinensis*, E6- *A. brasiliensis + G. lucidum*, E7- *A. brasiliensis + G. frondosa*, E8- *C. sinensis + G. lucidum*, E9- *C. sinensis + G. frondosa*, E10- *G. lucidum + G. frondosa*. Each value is expressed as means ± standard deviation (n=3). Means with different letters within a column are significantly different ($P<0.05$).

Almost all polysaccharides samples showed results close to controls of reducing power activity. Except polysaccharides of *A. brasiliensis* (P1) and *C. sinensis + G. frondosa* (P9) compared with others mushrooms and combinations. *G. lucidum* (P3), *G. frondosa* (P4) and *G. lucidum + G. frondosa* (P10) presented absorbance of 1.37 ± 0.122, 1.36 ± 0.015, 1.35 ± 0.015 respectively. These samples not showed differences statically compared with the control α-tocopherol. Exopolysaccharides of *C. sinensis* (P2), *A. brasiliensis + C. sinensis* (P5), *A. brasiliensis + G. lucidum* (P6), *A. brasiliensis + G. frondosa* (P7), *C. sinensis + G. lucidum* (P8) presented similar results with control BHA (Figure 4).
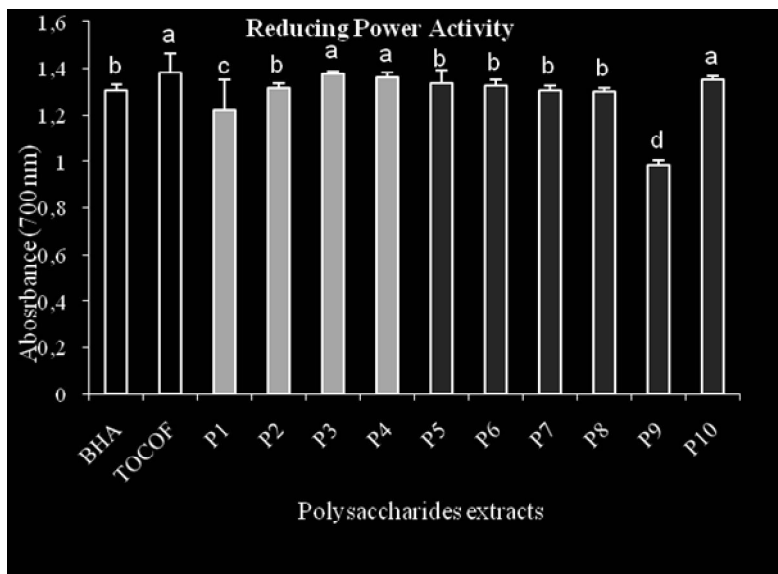
Figure 4: Reducing power of exopolysaccharides from mushroom cultivated by synergically submerged fermentation. Exopolysaccharides from mushroom produced by submerged fermentation: P1- *A. brasiliensis*, P2- *C. sinensis*, P3- *G. lucidum*, P4- *G. frondosa*, P5- *A. brasiliensis + C. sinensis*, P6- *A. brasiliensis + G. lucidum*, P7- *A. brasiliensis + G. frondosa*, P8- *C. sinensis + G. lucidum*, P9- *C. sinensis + G. frondosa*, P10- *G. lucidum + G. frondosa.* Each value is expressed as means ± standard deviation (n=3). Means with different letters within a column are significantly different ($P<0.05$).

Reducing capacity of a compound may serve as a significant indicator of its potential activity. In this assay, the yellow color of the solution changes to various shades of green and blue depending on the reducing power of each compound. The presence of reducing agents in the test solution results in reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Ferreira *et al*., 2007).

Antioxidant activity of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Jayakumar *et al*., 2007; Elmastas *et al*, 2007).

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3.4 Superoxide anion scavenging activity (O_2^-)

The best effect of samples of mushroom on superoxide radical was with ethanolic biomass extracts. However, the highest scavenging ability was exhibited by biomass of *A. brasiliensis* + *G. lucidum*, with 127.58 ± 2.4 following of *A. brasiliensis* + *C. sinensis* with 113.60% ± 6.69, *C. sinensis* + *G. frondosa*, 88.38% ± 7.27 and *A. brasiliensis* with 80.712% ± 1.269. *C. sinensis* isolated showed activity of 25.571% ± 0.679, but when grown synergistically with *A. brasiliensis* or *G. frondosa*, showed high scavenging activity. And curiously, the ethanolic biomass extracts of isolate cultivation of *G. lucidum* and *G. frondosa* were 8.26 ± 0.57 and 40.85 ± 2.25, respectively. And co-cultivation from *G. lucidum* + *G. frondosa* presented approximately the sum of both isolated, 48.36 ± 2.0 (Table 4):

Table 4: Determination of superoxide anion radical and hydroxyl radical scavenging activities

Mushrooms cultivated by submerged fermentation (%)			
A. brasiliensis (*Ab*)	*C. sinensis* (*Cs*)	*G. lucidum* (*Gl*)	G. *frondosa* (*G.f*)
EBE	80.71 ± 1.26	25.57 ± 0.67	8.26 ± 0.57	40.85 ± 2.25
BF	3.75 ± 0.46	3.75 ± 0.46	1.46 ± 0.90	2.36 ± 0.34
EPS	0.49 ± 0.01	2.69 ± 0.34	3.10 ± 0.32	3.64 ± 0.57

Mushrooms cultivated synergically by submerged fermentation					
Ab + Cs	*Ab + Gl*	*Ab + Gf*	*Cs + Gl*	*Cs + Gf*	*Gl + Gf*
EBE	113.6 ± 6.69	127.58 ± 2.4	18.44 ± 0.16	8.65 ± 0.71	88.38 ± 7.27	48.36 ± 2.0
BF	1.90 ± 0.67	2.28 ± 0.43	0.97 ± 0.02	0.16 ± 0.002	2.04 ± 0.15	1.30 ± 0.01
EPS	1.71 ± 0.11	3.50 ± 0.57	0.98 ± 0.003	1.63 ± 0.16	3.99 ± 0.57	1.57 ± 0.24

*(EBE) Biomass Ethanolic Extract; (BF) Fermented Broth and (EPS) Exopolysaccharide.

In submerged culture of *Antrodia camphorate* was affected scavenging free radical activity of both methanolic extracts of the mycelia and broth extracts. The optimal scavenging effects were 22% and 28%, respectively, for mycelium and filtrate at 0.2 mg/mL (Shu and Lung, 2008). Elmastas *et al.*, (2007) studied inhibition of superoxide generation of edible mushroom species and was found 99, 98, 97, 71 and 78

(%) for *Verpa conica*, *Boletus badius*, *Russula ostreatus*, *A. bisporus* and *Polyporus squamosus*, respectively.

The superoxide radical is known to very harmful to cellular components since it is precursor of more reactive oxygen species. Superoxide anion is a reduced form of molecular oxygen created by receiving one electron. Superoxide anion is an initial free radical formed from mitochondrial electron transport systems. Mitochondria generate energy using 4-electron chain reactions, reducing oxygen to water. Some of the electrons escaping from the chain reaction of mitochondria directly react with oxygen and form superoxide anion. It plays an important role in the formation of the other reactive oxygen species, such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems (Jayakumar *et al*., 2009; Siddhuraju and Becker, 2007).

3.5 Determination of total phenolics compounds

Comparatively, results from co-cultivation were better than isolated cultivation. The higher phenolic compounds counted of biomass found were *A. brasiliensis* + *G. lucidum* with 168.84 ± 10.49 µg/mL Gallic Acid Equivalents (GAE), *G. lucidum* + *G. frondosa* with 144.6 ± 4.18 µg/mL GAE and *C. sinensis* + *G. frondosa*, 143.3 ± 11.10 µg/mL GAE. Broth fermented samples from *A. brasiliensis* + *G. frondosa* presented 156.7 ± 3.63 µg/mL GAE and *G. lucidum* + *G. frondosa*, 131.5 ± 3.65 µg/mL GAE. Exopolysaccharide (EPS) in co-cultivation from *C. sinensis* + *G. frondosa* obtained 165.21 ± 7.56 µg/mL GAE (Table 5).

Table 5: Total phenolic compounds in EBE, FB and EPS of submerged fermentation of mushrooms (µg/mL gallic acid equivalent - GAE)

	Mushrooms cultivated by submerged fermentation (µg/mL GAE)			
	A. brasiliensis (*Ab*)	*C. sinensis* (*Cs*)	*G. lucidum* (*Gl*)	*G. frondosa* (*G.f*)
EBE	131.27 ± 7.27	111.87 ± 7.56	116.72 ± 3.63	131.87 ± 5.479
FB	132.48 ± 4.19	122.78 ± 10.49	107.03 ± 2.09	131.27 ± 3.63
EPS	94.90 ± 1.74	100.96 ± 4.19	108.24 ± 5.55	98.54 ± 3.63

	Mushrooms cultivated synergically by submerged fermentation					
	Ab + *Cs*	*Ab* + *Gl*	*Ab* + *Gf*	*Cs* + *Gl*	*Cs* + *Gf*	*Gl* + *Gf*
EBE	130.7 ± 10.2	168.8 ± 10.5	105.8 ± 3.6	102.1 ± 3.63	143.3 ± 11.1	144.6 ±4.2
FB	108.7 ± 3.6	124.0 ± 9.6	156.7 ± 3.6	114.3 ± 2.1	115.1 ± 5.5	131.5 ±3.6
EPS	107.0 ± 6.3	102.1 ± 3.6	90.0 ± 4.2	102.1 ± 3.6	165.2 ± 7.5	99.7 ± 4.2

*(EBE) Ethanolic Biomass Extract; (FB) Fermented Broth and (EPS) Exopolysaccharide.

Lactarius deterrimus, *Suillus collitinus*, *Boletus edulis* Bull., *Xerocomus chrysenteron* contain 8.6, 13.59, 31.64 and 17.91 µg/GAEs/mg extract of fruit body of mushroom (Sarikurkcu *et al*., 2008). According Turkuglu *et al*. (2007), BHA and α-tocopherol at 160 µg/mL concentrations showed 96.4% and 98.6% of oxidation inhibition and *Laetiporus sulphureus* (Bull.) Murrill extracts inhibited 82.2% in same concentration.

Fruit body of *Lentinus edodes* contains 29.0 mg/100g (GAE per 100 g of sample) and after heat treatment polyphenolics were increased to 54.6 mg/100 g (Choi *et al*., 2006). Lee *et al*. (2008) showed *Hypsizigus marmoreus* contents of antioxidant components 40.22 and 18.01 mg/g for ethanolic extracts from fruit bodies and mycelia and 10.29 and 16.39 mg/g hot water extracts, respectively.

Mushroom with high concentration of phenolic compounds show higher yield in the process of extraction with polar solvents. The addition of water in the extraction process of phenolic compounds increases the extract yields.

Total phenolic compounds may contribute directly to antioxidant action and play role in stabilizing lipid peroxidation. Phenolic compounds more studied are: caffeic acid, gallic acid and ellagic acid. These compounds are important for diet and may inhibit lipidic peroxidation process. It is suggest that high phenolic compounds contend can reduce the risk of heart disease by slowing the progression of atherosclerosis and

have inhibitory effects on mutagenesis and carcinogenesis in humans when up to 1.0 g is ingest daily from diet rich fruits and vegetables (Dubost and Beelman, 2007; Turkuglu *et al*., 2007; Wu *et al*., 2006).

The family of phenolic compounds in nature are just distributed a few of them, though they are found with some frequency. In this group are simple phenols, the pyrocatechol, hydroquinone and resorcinol. To this family also belong the aldehydes derived from benzoic acids, which are components of essential oils such as vanillin

4 Conclusion

According to the results of this study, the co-cultivation in submerged fermentation considered the combination *G. lucidum + G. frondosa* with higher activity for scavenging effect on DPPH and for reducing power activities. However, the isolated cultive of *G. lucidum* showed results close to this co-cultive. Biomass ethanolic extracts generally were more efficient than exopolysaccharides for DPPH activities.

A.brasiliensis + G. lucidum combination presented interesting results for superoxide anion scavenging activity and total phenolic compounds showed potential for antioxidant activity, mainly with biomass ethanolic extracts.

Lipid peroxidation by ß-caroten-linoeic acid systems presented lower activity compared with BHA and controls α-tocopherol. This indicate that mushroom tested have tendency for inhibition on transfer of electrons from antioxidant to oxidant. Our results suggest studying different solvents for biomass extracts and different concentrations. Biomass extracts, broth fermented and polysaccharides are possible food supplement or even as pharmaceutical agent by submerged fermentation. The combination among mushrooms cultivated by submerged fermentation may improve effects antioxidant.

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Chapter IV

ANTI-PROLIFERATIVE EFFECT ON TUMORAL CELLS OF EXOPOLYSACCHARIDES FROM *Agaricus brasiliensis* PRODUCED SYNERGICALLY BY SUBMERGED FERMENTATION WITH *Cordyceps sinensis*, *Ganoderma lucidum* AND *Grifola frondosa*

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Abstract

Mushroom has been used as a health food due to production of several bioactive compounds, especially polysaccharides. The present study aimed to evaluate anti-proliferative tumor cells activity of exopolysaccharide, produced by submerged fermentation of mushroom. The species fermented were: *Agaricus brasiliensis, Cordyceps sinensis, Ganoderma lucidum* and *Grifola frondosa* with respective association: *A. brasiliensis + C. sinensis* (Ab + Cs), *A. brasiliensis + G. lucidum* (Ab + Gl) and *A. brasiliensis + Grifola frondosa* (Ab + Gf). The synergic fermentation showed lower yield in biomass growth and EPS production when compared with isolate species. When it comes to anti-proliferative activity against Sarcoma 180 cells, EPS produced by co-cultive presented better results compared with EPS produced by controls. For treatment for Ehrlich tumor, this is not observed. In the cell viability test, the EPS of mushroom, at 500 μg/well in microplate, on macrophages not exhibited toxic effect on macrophages. Monosaccharide composition present in the EPS of Ab + Cs were mannose (38.21%), followed by galactose (48.44%) and glucose (5.45%), and lower amounts of fucose (3.5%). EPS produced by Ab + Gl contained 87. 4% of galactose; and Ab + Gf showed 50.5% of mannose and 30.4% of galactose. For the EPS majority sugar contends of Ab + Gf and Ab + Gl were 51% of mannose and 88% of galactose, respectively.

Key-words: Mushroom, submerged fermentation, exopolysaccharides, tumor cells.

1 Introduction

The microbial polysaccharides (EPS) are a class of high value biopolymers with a wide variety of industrial and pharmaceutical applications. Bioactivity of polysaccharides from Chinese traditional medicine, especially from fungi, has been investigated for many years. Some of fungus polysaccharides are use for diseases treatment and biological tonic, anti-ageing and others applications (Wasser *et al.*, 2002).

Polysaccharides can be obtained directly from fruiting bodies or from mycelia. However, researchers showed the superiority of liquid culture over fruiting body cultivating, such as low-cost, short-cycle life and year round productivity (Angeli *et al.*, 2009, Gao and Gu, 2007, Cho *et al.*, 2006; Wu *et al.*, 2003; Kurbanoglu, Algur and Zulkadir, 2004).

Optimizing the production of exopolysaccharides and others compounds with higher biological activity has been objective of study for many researchers.

Agaricus brasiliensis, *Cordyceps sinensis, Ganoderma lucidum and Grifola frondosa* are mushrooms species used with medical application, mainly in Asiatic countries.

The Basidiomycete *Agaricus brasiliensis,* an edible mushroom belonging to Agaricaceae family, is native to southern Brazil and was introduced from Japan around 1950. Popularly known as "Himematsutake" or "Sun Mushroom", have medicinal potential, such as antioxidants, immunomodulatory, hypocholesterolemic, hypoglycemic and anti-activity cancer (Angeli *et al.*, 2009; Gao and Gu, 2007, Mizuno *et al.*, 1999).

Cordyceps is belongs to Phylum Ascomycota classified in the Clavicipitaceae. This mushroom is available as Chinese herbs with anti-aging, ''pro-sexual", anti-cancer effects. *C. sinensis* species are parasites of insects or fungi, often exhibiting a high degree of host specificity and some species are used as insect bio-control agents (Paterson *et al.,* 2006; Cha *et al.*, 2005; Huang *et al.*, 2006).

G. lucidum (Fr.) Krast (Polyporaceae) also called "Lingzhi", contains a variety of chemical substances. The fruit body of *G. lucidum* is a large, dark mushroom and no documented toxicity (Seto *et al.*, 2009). It is used as health-promotion supplement, anti-tumor and immuno-modulating effects. The abilities of liver protection, hypoglycemia, hypertension, and neoplasia inhibition have also been demonstrated from the fruiting

bodies and cultured mycelia (Chen *et al*., 2004). The polysaccharides of *G. lucidum* are the major source of its biological activity and therapeutic uses (Hsieh *et al.,* 2006).

G. frondosa is a Basidiomycete fungus belonging to the order Aphyllopherales, and the family Polyporaceae. Studies medicinal effects of *G. frondosa* include anti-cancer, immunostimulation, anti-angiogenesis, antibacterial and antiviral effects, vitality, antioxidant effects, and beneficial cosmetic effects on skin (Yang *et al*., 2007).

The aim of the present study was evaluate anti-tumoral property of the exopolysaccharide, produced by submerged fermentation of mushroom, in association with others species, against tumor cells *in vitro* and partial characterization of exopolysaccharide.

2 Material and Methods

2.1 Mushrooms

The strain of *Agaricus brasiliensis, Cordyceps* sinensis, *Ganoderma lucidum* and *Grifola frondosa* were obtained from the standard stock of Bioprocesses and Biotechnology Laboratory (LPB) at Federal University of Paraná (UFPR), Curitiba-Brazil. They were maintained on potato-dextrose agar, incubated at 30ºC ± 2 for seven days followed by refrigeration at 4ºC. The association among mushrooms for submerged fermentation was determined according with table 1, with respective controls:

Table1: Mushroom of submerged fermentation and associations for EPS production:

Mushroom (Controls)		Synergic fermermentation of mushrooms	
Agaricus brasiliensis	(Ab)	*Agaricus brasiliensis + Cordyceps sinensis*	(Ab + Cs)
Cordyceps sinensis	(Cs)	*Agaricus brasiliensis + Ganoderma lucidum*	(Ab + Gl)
Ganoderma lucidum	(Gl)	*Agaricus brasiliensis + Grifola frondosa*	(Ab + Gf)
Grifola frondosa	(Gf)		

2.1.1 Pre-Inoculum preparation

Mushrooms were initially grown on PDA medium in a Petri dish with 90 mm of diameter, and then the mycelium was transferred to 500 mL flasks, containing 250 mL of basal modified medium composed (g/L^{-1}): glucose, 20, yeast extract, 3.0, $MgSO_4.7H_2O$, 0.3 and $K_2HPO_4.3H_2O$, 0.6; and pH 6.1, according Fan *et al.*, (2005), modified. The flask was incubated under agitation of 120 rpm, 28ºC ± 0.2, during 10 days.

2.2 Submerged Fermentation

The submerged fermentation was conducted in flasks of 1 L contain 500 mL of basal medium by Fan *et al*. (2005). The inoculum ratio was 4% (v/v) prepared for each mushroom, totalizing 8% (v/v) for each flask. The fermentation was carried out at 28°C, agitation of 120 rpm, initial pH 6.1 during 10 days. Aliquots were taken daily for kinetic analyses. The isolate species (control) were cultivated individually submerged fermented in the same conditions.

2.3 Exopolysaccharides extraction

The culture was filtered using Whatman 1 filter paper, under low pressure. The filtrate was concentrated to ¼ of the original volume by rotary evaporator under reduced pressure at temperature below 50 °C. In the concentrated sample was added four parts of 95% ethanol at low temperature (4°C) overnight for EPS precipitation (Gonzaga *et al*., 2005, Rubel *et al*., 2008). The precipitated was obtained by centrifugation at 4000 rpm for 20 min and washed twice with ethanol. Afterward, distilled water was added and only the soluble fraction was used for tests. The samples were filtrated in 0.22 μm filter for anti-proliferative tumor cells tests.

2.4 Analytical Methods of kinetics

The exopolysaccharide (EPS) was determined by phenol-sulfuric acid method. Residual glucose was measured according to Somogyi-Nelson (1945). The biomass was measured by dry weight (g/L) and pH verified in pHmeter.

2.5 Biological Assays

2.5.1 Anti-proliferative activity on Ehrlich tumor and Sarcoma 180 cells *in vitro*

2.5.1.1 Tumor Cells Culture

A single cell suspension of Ehrlich tumors cells and Sarcoma 180 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine, and 1% antibiotics, and incubated at 37°C in humidified incubator contain 5% CO_2.

The cell concentration was adjusted to 1.0×10^5 cells/mL and distributed in a 96 well-plate. The microplate was incubated for 24 h and different concentrations (10, 30, 60, 100, 150, 300 and 500 μg/ml) of EPS from associated mushrooms were added. The cultures were incubated kept for 48 h at 37 °C in incubator, maintaining a constant atmosphere of 5% CO_2. PBS (phosphate buffer solution, 0.2 M, pH 7.4) was used to the negative control group. Proliferation was checked by MTT assay method. All tests were carried out in triplicate. The results were compared to the controls groups. Percentage of Ehrlich tumor cells and Sarcoma 180 cells the inhibitory rate were calculated as follows:

$$Inhibitory\ rate\ (\%) = \left[(B - A)/B\right] x\ 100\%$$

Where, *A* is the average absorbance at 550 nm of treated groups and *B* is the average absorbance at 550 nm of untreated group (control group).

2.5.1.2 Citotoxicity of macrophages "in vitro"

Peritoneal macrophages were isolated from female Wistar mice with 20 days age intraperitoneally injected with 10 ml of ice-cold PBS. The macrophages were harvested by peritoneal washed, and the cells were subsequently incubated in 96-well plate containing RPMI supplemented with 10 % fetal bovine serum (Cultilab, Materials for Cell Culture Ltda., Campinas, SP, Brazil), 10 μg/ml streptomycin and 10 IU/ml penicillin (Gibco, Invitrogen Corporation, Grand Island, NY, USA). Peritoneal macrophages were propagate in 3.0×10^5 (cells/well). EPS were added to wells in

different concentrations (5, 10 and 20 mg/ml). The cells were cultured for 24 hours at 37°C, 5% CO_2. The viability of macrophages was checked by MTT assay method. All tests were carried out in triplicate. Cell viability of macrophages was calculated:

$$Cell\ viability\ (\%) = [(B - A)/B] \times 100.$$

Where A is the average absorbance at 550 nm of treated groups and B is the average absorbance at 550 nm of untreated group (control group). Ethic committee, register: nº 213 and process: 23075.013165/2007-58.

2.6 Monosaccharide composition

Approximately 1 mg of EPS was treated with 0.5 ml of trifluoracetic acid (TFA), 1 M, for 1 hour and 120°C. The acid was eliminated by evaporation until dissect, followed by sodium borohydride ($NaBH_4$) reduction, acetylation and analyzed by Gas chromatography (CG) (Lima *et al*., 2007).

2.7 Statistical analysis

Data were expressed as means ± SD and were analyzed statistically with ANOVA. The level of significance was at a P value less than 0.05. Data in all the bioassays were statistically evaluated by Tukey's test by variance analysis and $P < 0.05$ was considered significant.

3 Results and Discussion

3.1 Kinetics of growth of synergic fermentation of mushrooms

In submerged fermentation of mushroom, the basic variables for fermentation monitoring were pH, glucose, biomass and EPS production. Submerged fermentation values are the mean of triplicate independent experiments and calculated the standard deviation.

During the submerged fermentation of mushrooms, pH initial 6.1, was not controlled. The results demonstrated that a lower pH at early fermentation stage was advantageous to cell growth by glucose consumption rate. The synergic fermentation of Ab + Cs reached pH 3.9 at 5-day, while the isolate species presented *A. brasiliensis,* 4.8 and *C. sinensis* was 3.7. Samples Ab + Gl and Ab + Gf both showed pH 4.7, at 2-day and 4-day, respectively (Figure 1). After 8-10-day of fermentation, the pH elevated for all samples. *A. brasiliensis* reached pH 7.0 and Ab + Gl showed similar results, pH 6.9 (Table 2). The change pH is directly proportional to the concentration of glucose in the medium during fermentation.

Mushroom uses glucose as the main source of carbon and energy for its growth. Consuming glucose, the fungi produces acids as part of its energy metabolism, increasing the acidity of the medium, and this fact may affect cell membrane function, cell morphology and structure, the uptake of various nutrients, and product biosynthesis.

Glucose consumption ranged 29.5 to 76% in samples of fermented mushrooms. The initial amount of glucose was 20 g/L. At 10-day fermentation, the synergic fermentation of mushroom Ab + Gl and Ab + Gf showed 60.5% ± 0.65 and 76% ± 6.5 consume of glucose (Table 2). The incomplete utilization of reducing sugars by the fungus in media suggested that sugars concentrations play a role in EPS production but also the water activity might be involved (Pappinuti, 2010).

G. lucidum presented higher production of biomass (10.9 g/L ± 0.3595) at 10-day and EPS (1.827 g/L ± 0.0830) at 7-day. The co-cultive Ab + Gl showed biomass production of 6.74 g/L ± 0.394 and EPS production of 1.30 g/L ± 0.029 (g/L) (Table 2). The combination of mushroom showed lower yield in biomass growth and EPS production when compared with isolate species. The EPS production by synergic

fermentation was lower than isolate cultive. This fact may be cause suppose competition among mushroom species. Data demonstrate that EPS is a metabolic product of log phase.

Table 2: Values obtained of submerged fermentation of mushrooms during 10 days

Mushrooms	Glucose consume (%)	pH (min.)	Biomass (g/L)	EPS (g/L)	EPS yield/day
A.brasiliensis	58.0 ± 1.02	4.5 ± 0.02	8.65 ± 0.09	1.69 ± 0.02	0.28
C. sinensis	50.5 ± 3.4	3.7 ± 0.07	9.68 ± 0,5	1.01 ± 0.02	0.20
G. lucidum	35.5 ± 1.5	4.1 ± 0.02	10.04 ± 0.35	1.82 ± 0.08	0.26
G. frondosa	29.5 ± 3.5	5.4 ± 0.11	2.6 ±0,03	1.07 ± 0.02	0.17
Co-cultivation					
Ab+Cs	48,5 ± 3.9	3.9 ± 0.04	1.3 ± 0.02	1.17 ± 0.01	0.19
Ab+Gl	60.5 ± 0.65	4.7 ± 0.1	8.35 ± 0.84	1.30 ± 0.02	0.21
Ab+Gf	76.0 ± 6.5	4.7 ± 0.04	5.5 ± 0.38	1.34 ± 0.01	0.33

Cui *et al*. (2006) demonstrated exopolymer production of *G. frondosa* was 1.326 g/L in bioreactor 15 L. And Lee *et al*. (2007) obtained 1.6 g/L from *Ganoderma applanatum.*

According Pappinuti (2010), the EPS production by *G. lucidum* presented different yields in different culture conditions and glucose presented the more efficient enhancers of EPS production. Zou (2006) suggests that relativity higher initial glucose concentration led to higher production of EPS by *A. brasiliensis.*

Xiao *et al*. (2010) studied *Cordyceps taii*, a new medicinal mushroom, to identify an optimum carbon source for EPS production. It was tested six monosaccharides, three disaccharides, honey sugar and soluble amylum in submerged culture. The best result was xylose who yielded the highest level of EPS, and whose production was more than 3-fold greater than in basal medium containing glucose.

Chen *et al*. (2008), in study with *Fomes fomentarius*, a high level of mycelial biomass and EPS was obtained when glucose, mannitol, galactose was used as the carbon source.

3.2 Biological assays

3.2.1 Anti-proliferative activity of EPS on Ehrlich tumor and Sarcoma 180 cells

According to the obtained results, Ehrlich tumor and Sarcoma 180 cells treated with exopolysaccharides exhibited significant inhibitory ratios in proliferation activity at all concentrations.

EPS produced by *A. brasiliensis* showed more efficient than EPS produced by co-cultivation with mushrooms for treatment Ehrlich tumor cells *in vitro*.

Ehrlich tumor cells were inhibited 60.12% ± 2.181 at 500 µg/well of EPS produced by *A. brasiliensis*.

EPS produced in co-culture showed Ehrlich tumor cells anti-proliferative activities with lower concentrations compared with *A. brasiliensis*, isolated cultivation. Therefore, Ab + Gl showed around 50% of proliferation inhibition at 30 – 150 µg/well, there is no significant difference. Concentrations at 300 -500 µg/well of Ab + Gl not showed the same effectiveness. And EPS produced from Ab + Cs showed proliferation inhibition of 52.6% ± 3.956 in the 500 µg/well concentrations. The EPS of Ab + Gf presented around 50% of Ehrlich tumor cells anti-proliferative activity, no presented significant differences in EPS concentrations at 10-150 µg/mL, according with test ANOVA ($p < 0,05$) (Figure 1).

Figure 1: Inhibition of Ehrlich tumor cells proliferation by exopolysaccharides at different concentrations *in vitro*. Each bar represents the mean SD. Different symbols indicate different statistical groups by Tukey´s test at 95% of significance, n =5, *P*<0.05.

EPS *A. brasiliensis* inhibited cell proliferation of Sarcoma 180 in 51.47% ± 4.13, however with 500 µg/mL. Data with EPS *A. brasiliensis* were dose-dependent. The inhibition of Sarcoma 180 cells proliferation of with EPS of Ab + Cs was 54.3% ± 0.140 at 30 – 500 µg/mL. This EPS showed better inhibition with lower concentration compared with others EPS tested. Co-cultivation of Ab + Gl, was 54.5% ± 1.03 of inhibition of proliferation of Sarcoma 180 cells at 300 – 500 µg/mL concentration. And this EPS reached 44.03% ± 3.07 of inhibition proliferation at 10 µg/mL concentration. The EPS of Ab + Gf showed inhibition of tumor cells was around 53.0% at 100 - 300 µg/mL, no presented significant differences in EPS concentrations, according with test ANOVA (p<0,05) (Figure 2).
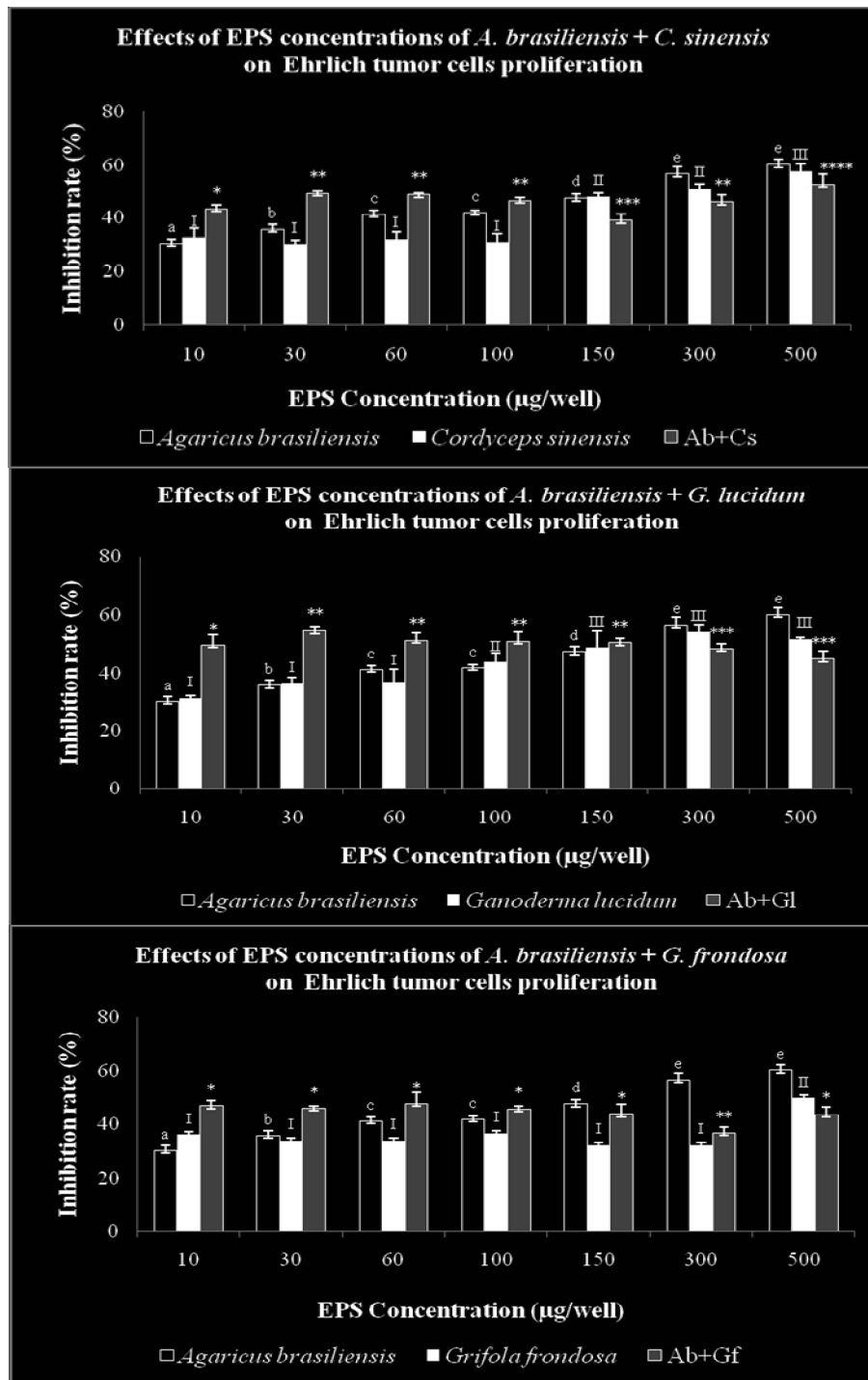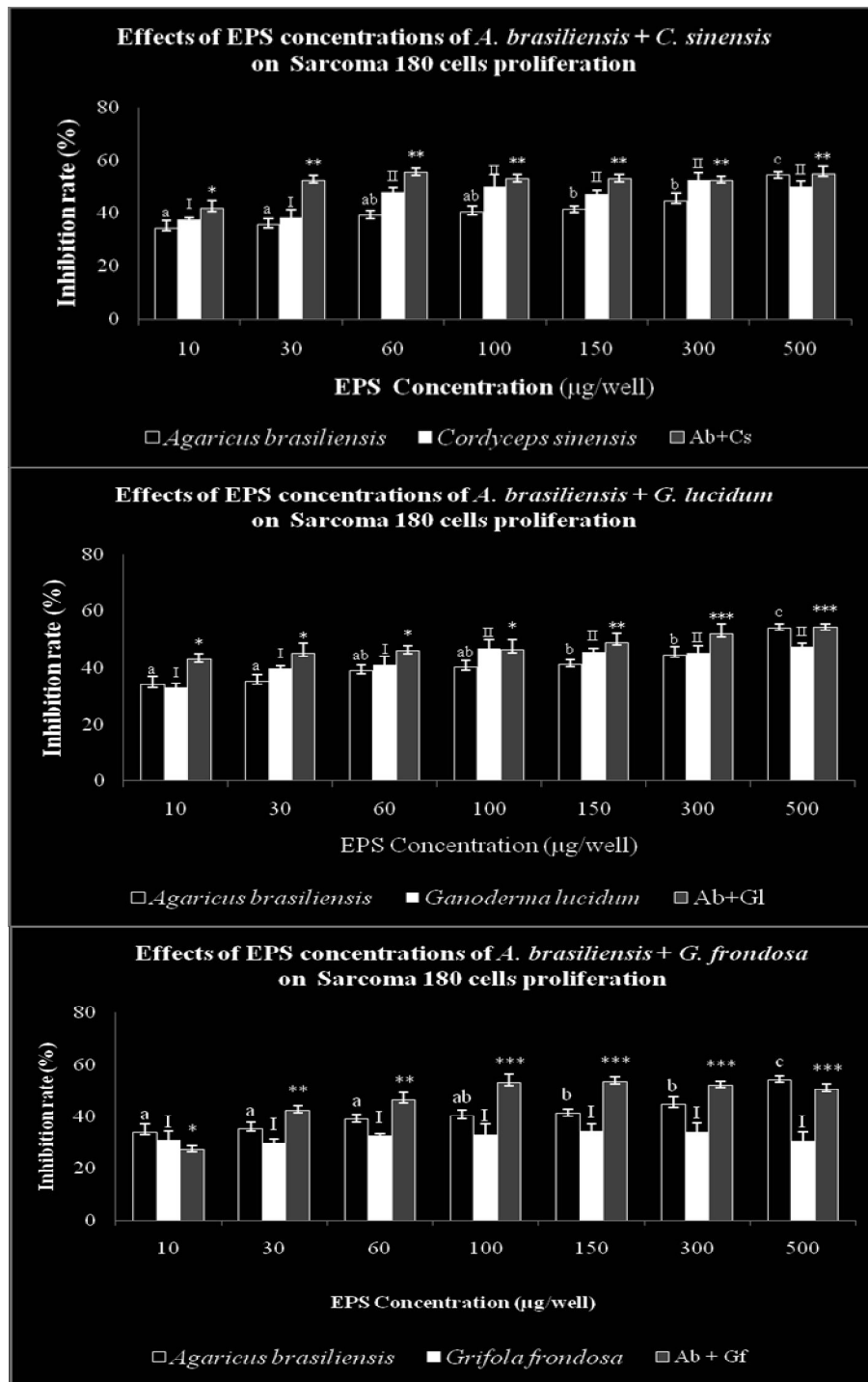
Figure 2: Inhibition of Sarcoma 180 cells proliferation by exopolysaccharides at different concentrations *in vitro*. Each bar represents the mean SD. Different symbols indicate different statistical groups by Tukey´s test at 95% of significance, n =5, *P*<0.05.

The anti-proliferative activity of EPS against tumor cells was different for each tested type. For Sarcoma 180 cells treatment, EPS produced by co-cultive presented higher results of anti-proliferative activity compared with EPS produced by controls (species cultivate isolate). For Ehrlich tumor treatment, this is not observed.

The proposed mechanisms by which mushroom polysaccharides exert antitumor effect include:

1) cancer-preventing activity: the prevention of the oncogenesis by oral administration of polysaccharides;

2) Immune-enhancing activity: enhancement of immunity; and

3) Direct tumor inhibition activity: direct antitumor activity to induce the apoptosis of tumor cells.

Studies indicate that incubation of polysaccharides together with tumor cells could change the expression of signals within tumor cells. That could arrest the cell cycle and generate apoptosis, which explains the *in vitro* anti-proliferative effect of polysaccharides (Zhang, *et al.*, 2007).

Sarangi *et al.* (2006) demonstrated that two fractions of *Pleurotus ostreatus* can directly kill Sarcoma 180 cells *in vitro*. According Wu *et al.*, 2006, cell-cell adhesion determines the polarity of cells and participates in the maintenance of the cell societies called tissues. Adhesion is generally reduced in human cancer cells. Reduce intercellular adhesion allows cancer cells to disobey the social order, resulting in the destruction of histological structure, which is the morphological hallmark of malignant tumors. Reduced intercellular adhesiveness is also indispensable for cancer invasion and metastasis.

Exopolysaccharides were mainly used as adjuvant in post-operative treatment chemotherapy, radiotherapy, and radiochemotherapy and endocrine therapy of different cancers such as breast cancer, cervical cancer, colorectal cancer, gastrointestinal cancer, lung cancer and prostate cancer. The clinical trials demonstrated the polysaccharide can prolong the survival and improve the quality of life of the patients. The parameters employed for measurement of quality of life included appetite, sleep, nausea/vomiting, and abdominal pain/diarrhea. The prolongation of patient's survival was linked to increased infiltration of T cells, B cells and macrophages in tumor prevention of

metastasis and restoration of T-helper 1 to T-helper 2 balance (Leung *et al.*, 2009, Dalla Santa *et al.*, 2009).

In experiments *in vivo*, studies of antitumor activities on Sarcoma 180 and Ehrlich carcinoma have showed great antitumor activity to regression tumors growth. The purified polysaccharide has been shown in animal studies to produce strong tumor regression and even the disappearance of sarcoma tumors in 5 weeks, ascite hepatoma 134, and Ehrlich carcinoma as well as a number of other experimentally induced cancers (Moradali *et al.*, 2007).

3.3 Cytotoxicity macrophages assay

Macrophages, which are part of innate immune system, play an essential and pivotal role in protecting our body from any type of invading cells including cancer cells. ß-glucans bind to toll-like receptors on macrophage and trigger activation process (Sarangi *et al.*, 2006). The EPS produced in co-cultive were tested on macrophages, by toxicity (Figure 3). An MTT assay has shown indirect cell-mediated cytotoxicity based on hydrolysis by mitochondrial dehydrogenases of living cells.
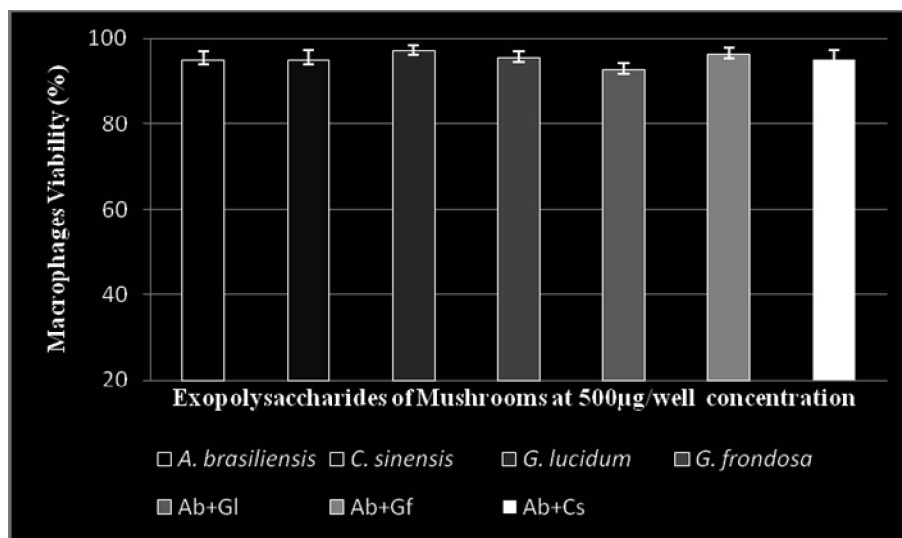

Figure 3: Effect of exopolysaccharides at 500 μg/well concentrations on mice macrophages proliferation *in vitro*. Values are means ± SD of triplicates, n =5, *P*<0.05.

EPS produced by submerged fermentation synergic, at 500 μg/well, on macrophages not exhibited toxic effect on macrophages. The control with macrophages and RPMI media was considered 100% of viability cells. All treatments presented values higher 90% of viability of cells. Ab + Gf obtained 91.6% ± 1.23 of viability cells, the lower results.

ß-glucans and α-mananas are usually strong mitogens. Macrophages, T-lymphocytes, B-lymphocytes and NK cells proliferate in response to polysaccharide stimulation, which is mediated through the biding of polysaccharides to its corresponding receptors. The mitogenic activation macrophages, B-lyphocytes and NK cells by polysaccharides are characterized by usage of mitogen-activated protein kinases in the intracellular signaling events (Leung *et al*., 2006).

3.4 Monosaccharidic composition

Data are restricted to polysaccharide with simple monosaccharide composition and glycosidic linkages like ß-glucanas and manan, and most of the researches were perfomed on ß-glucan. The mushroom derived polysaccharides with anticancer effects vary from being homopolymers to highly heteropolymers. Differences in activities are generally related with solubility in water, molecular size, branching frequency and forms (Sarangi *et al*., 2006).

EPS production of mushrooms by synergic fermentation presented interesting results. *A. brasiliensis* was present for all synergic fermentations but the monosaccharide composition was different. The synergic growth of mushrooms may influence metabolic activity and produce news compounds.

These analyses revealed in the molar percentage that the principal sugars present in the EPS of Ab + Cs were mannose (38.21%), followed by galactose (48.44%) and glucose (5.45%), and lower amounts of fucose (3.5%). EPS produced by Ab + Gl contained 87. 4% of galactose; and Ab + Gf showed 50.5% of mannose and 30.4% of galactose (Table 3). Majority sugar contends of EPS of Ab + Gf and Ab + Gl were 51% of mannose and 88% of galactose, respectively.

Chen and Seviour (2007) analyzed the polysaccharides from the medicinal fungi, including *A. brasiliensis* and found that myo-inositol, sorbitol, fucose, galactosamine, glucosamine, galactose, glucose, and mannose were the neutral sugars in these

polysaccharides, and fructose, glucose and mannose were the predominant monosaccharides from this fungus.

Table 3: Composition monosaccharide of EPS of mushrooms by Gas Chromatographic

Composition monosaccharide (%)					
Mushroom	Rhamnose	Fucose	Mannose	Galactose	Glucose
A. brasiliensis	1,5	2,5	45,0	35,0	14,0
C. sinensis	0,5	0,5	64,0	7,0	27,0
G. lucidum		1,0	26,0	14,0	59,0
G. frondosa	0,4	0,2	4,3	0,6	93,7
Ab + Cs	-	3,0	40,0	50,0	6,0
Ab + Gl	0,6	2,5	8,0	88,0	0,3
Ab +Gf	0,5	5,5	51,0	32,0	10,0

More than 100 types of polysaccharides with biological activities have been isolated from the fruit body and mycelia of *G. lucidum*. The structure of β-D-glucans has a relationship with the binding characteristics with receptors. Differences in activities are generally related with solubility in water, molecular size, branching frequency and forms. It is believed that structural features such as β (1→3) linkage in the main chain of the glucan and additional β (1→6) branch points are needed for anticancer action (Moradali *et al*., 2007; Sarangi *et al*., 2006)

4 **Conclusions**

Kinetic of submerged fermentation of co-cultivation and respective controls presented important information about EPS and biomass production. The combination Ab + Gf showed high glucose consume. However, the lower pH was isolate cultivation of *C. sinensis*. And the isolate cultivation of *G. lucidum* showed better results for production of biomass and EPS.

Anti-proliferative tumor cells activity showed effective with *A. brasiliensis* EPS in maximum concentration tested. Therefore, co-cultivation EPS showed inhibitory proliferation activity with lower concentrations than isolated cultivation. Ehrlich tumor cells proliferation were inhibited by Ab + Cs and Ab + Gl EPS treatments. And Sarcoma 180 cells were inhibited by Ab + Cs and Ab + Gf EPS. In cell viability tests, all samples no showed cytotoxic effect in macrophages cells.

Composition monosaccharide was different for all samples. Co-cultivation method produced new compositions for each EPS.

Further studies are warranted to completely identify the real importance of these mushrooms as anticancer agent.

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Chapter V

ANTI-PROLIFERATIVE EFFECT ON TUMORAL CELLS OF EXOPOLYSACCHARIDES PRODUCED SYNERGICALLY BY SUBMERGED FERMENTATION WITH *Cordyceps sinensis*, *Ganoderma lucidum* AND *Grifola frondosa*

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Abstract

Bioactive exopolysaccharides have been isolated from mushrooms and these compounds have attracted significant attention because of their antitumor effects. The medicinal mushrooms, *Cordyceps sinensis, Ganoderma lucidum, Grifola frondosa*, were submerged fermented with two different species of mushroom in association for exopolysaccharides (EPS) production. The combinations of mushroom were: *Ganoderma lucidum* (Gl) + *Cordyceps sinensis* (Cs); *Ganoderma lucidum* (Gl) + *Grifola frondosa* (Gf); *Cordyceps sinensis* (Cs) + *Grifola frondosa* (Gf). The growth of mushroom was evaluated by kinetics. The pH change during the entire period was marked by glucose consumption which was used for biomass production. The co-cultivation Cs + Gf showed higher glucose consumption (73%) and biomass production of 4.4 g dw/L ± 0.212. Results of EPS production were: 1.09 g/L ± 0.803 (Gl + Cs) at the 4th day; 1.23 g/L ± 0.447 (Gl + Gf) at the 7th day and 1.03 ± 0.047 (Cs + Gf) at the 7th day. For anti-proliferative tumor cells properties, EPS Gl + Gf and Gl + Cs showed higher activity against Ehrlich tumor cell with 83.29% ± 3.53 at 500 (µg/well) and 60.6% ± 0.955 at 10 (µg/well), respectively. Cytotoxic test showed relatively low toxicity on macrophages. Sarcoma 180 cells were inhibited by isolated cultures around 50% cell proliferation, therefore, the co-cultivation no present results higher than controls. And monosaccharide composition of Gl + Cs presented glucose (71%) and mannose (18.5%) and of Gl + Gf contain 43.5% of glucose and mannose.

Key-Words: Mushroom, submerged fermentation, exopolysaccharides, tumor cells

1 Introduction

The number of mushroom species on earth is estimated at 140.000, yet maybe only 10% are known. Several bioactive compounds in medicinal mushrooms have been studied to benefit a wide range of diseases, including cancer, arthritis, heart diseases, cholesterol, immunologic system and others (Wasser *et al.*, 2002).

The three medicinal mushrooms: *Cordyceps sinensis, Ganoderma lucidum* and *Grifola frondosa* are known for biological properties and used in Asiatic countries. Medicinal mushrooms contain a high density of polysaccharides and triterpenes and over 1,000 other bioactive compounds. *C. sinensis* or Dong-Chong-Xia-Cao (winter worm–summer grass) in Chinese is a parasitic fungus growing on the larvae of the sphinx moth. This mushroom has been used for fatigue, night sweating, hyposexualities, hyperglycemia, hyperlipidemia, asthemia after severe illness, respiratory disease, renal dysfunction and renal failure, arrhythmias and other heart disease, and liver disease, as a tonic for longevity, endurance and vitality for thousands of years (Paterson, 2008; Wu *et al.*, 2007, Chen *et al.*, 2006, Li *et al.*, 2006; Yang *et al.*, 2005). *G. lucidum* is a favorite remedy in oriental medicine for centuries. Its fruiting body is called "Lingzhi" in China and "Reishi" in Japan. The major bioactive components in *G. lucidum* are polysaccharides, ganoderic acid (triterpene), and adenosine. *G. lucidum* is used as a supplement or for an alternative therapy (Cao *et al.*, 2006; Wu *et al.* 2006 and Xie, 2006).

G. frondosa are one traditional and edible mushroom in Southeast Asia, is commonly used in the treatment of various diseases, due to its considerable biological activities, such as hepatitis, hepatopathy, hypertension, nephritis, bronchitis, and cancers (Shi *et al.*, 2008, Kodama *et al.*, 2005 and Sliva *et al.*, 2002).

Chemical modification is often carried out to improve the biological selectivity and activity of polysaccharides and their clinical qualities by making them water soluble (Shi *et al.*, 2008). Submerged fermentation with two different species of mushroom in association has possibility to modify or produce new compounds. This study investigated the kinetics of mushrooms growth, the monosaccharide composition and anti-proliferative tumor cells activity of three medicinal mushrooms.

2 Materials and Methods

2.1 Mushrooms

The strains of *Cordyceps* sinensis, *Ganoderma lucidum* and *Grifola frondosa* were obtained from the standard stock of Bioprocesses and Biotechnology Laboratory (LPB) at Federal University of Paraná (UFPR), Curitiba-Brazil. They were maintained on potato-dextrose agar (PDA), incubated at 30ºC $\pm$ 2 for seven days followed by refrigeration at 4ºC. The association between two mushrooms strains in submerged fermentation was determined according to table 1:

Table1: Mushroom of submerged fermentation and associations for EPS production

Mushroom (Controls)		Synergic fermermentation of mushrooms	
Cordyceps sinensis	(Cs)	*G. lucidum+C. sinensis*	(Gl + Cs)
Ganoderma lucidum	(Gl)	*G. lucidum+G. frondosa*	(Gl + Gf)
Grifola frondosa	(Gf)	*G. frondosa + C. sinensis*	(Gf + Cs)

2.1.1 Pre-Inoculum preparation

Mushrooms were initially grown on PDA medium in a Petri dish with 90 mm of diameter, and then the mycelium was transferred to 500 mL flasks, containing 250 mL of basal modified medium composed (g/L^{-1}): glucose, 20, yeast extract, 3.0, $MgSO_4.7H_2O$, 0.3 and $K_2HPO_4.3H_2O$, 0.6; and pH 6.1, according Fan *et al*., (2005), modified. The flask was incubated under agitation of 120 rpm, 28ºC $\pm$ 0.2, during 10 days.

2.2 Submerged Fermentation

Submerged fermentation was carried out in 1 L flasks containing 500 mL of basal medium defined by Fan *et al*. (2005). The inoculum ratio was 4% (v/v) for each mushroom, totalizing 8% (v/v) for each flask. Fermentation was carried out at 28°C,

agitation of 120 rpm, initial pH 6.1 during 10 days. Aliquots were taken daily for kinetic analyses. The culture was filtered using Whatman 1 filter paper, under low pressure. The isolate species (controls) were submerged fermented in same conditions.

2.3 Exopolysaccharides extraction

The filtrate was concentrated to ¼ of the original volume by rotary evaporator under reduced pressure below 50 ℃. In the concentrated sample was added four parts of 95% ethanol at low temperature (4°C) overnight for EPS precipitation (Rubel *et al.*, 2010; Gonzaga *et al.*, 2005). The precipitated was recovered by centrifugation at 4000 rpm for 20 min and washed twice with ethanol. Afterward, distilled water was added and only the soluble fraction was used for tests. The samples were filtrated in 0.22 μm filter for anti-proliferative tumor cells tests.

2.4 Analytical Methods of kinetics

The exopolysaccharide (EPS) was determined by phenol-sulfuric acid method. Residual glucose was measured according to Somogyi-Nelson method. The biomass was measured by the method dry weight (g/L) and pH was analyzes in pHmeter.

2.5 Biological Assays

2.5.1 Anti-proliferative activity on Ehrlich tumor and Sarcoma 180 cells *in vitro*

2.5.1.1 Tumor cells culture

Ehrlich tumors cells and Sarcoma 180 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine, and 1% antibiotics, and incubated at 37℃ in humidified incubator containing 5% CO_2.

The cell concentration was adjusted to 1.0 x 10^5 cells/mL and distributed in a 96 well-plate. The microplate was incubated for 24 h and different concentrations (10, 30, 60, 100, 150, 300 and 500 μg/ml) of EPS from mushrooms associated were added. The cultures were kept for 48 h at 37 °C in incubator, maintaining a constant atmosphere of

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5% CO_2. To the positive and negative controls were used gallic acid (10%) and PBS, respectively. Proliferation was checked by MTT assay method. All tests were carried out in triplicate. The results were compared to the controls groups. Percentage of Ehrlich tumor cells and Sarcoma 180 cells the inhibitory rate were calculated as follows:

$$Inhibitory\ rate\ (\%) = [(B - A)/B]\ x\ 100\%$$

Where, A is the average absorbance at 550 nm of treated groups and B is the average absorbance at 550 nm of untreated group (control group).

2.5.1.2 Citotoxicity of macrophages "in vitro"

Peritoneal macrophages were isolated from female Wistar mice with 20 days age intraperitoneally injected with 10 ml of ice-cold PBS (phosphate buffer solution, 0.2 M, pH 7.4). The macrophages were harvested by peritoneal washed, and the cells were subsequently incubated in 96-well plate containing RPMI supplemented with 10 % fetal bovine serum (Cultilab, Materials for Cell Culture Ltda., Campinas, SP, Brazil), 10 µg/ml streptomycin and 10 IU/ml penicillin (Gibco, Invitrogen Corporation, Grand Island, NY, USA). Peritoneal macrophages were seemed in (3.0 x 10^5 cells/well) and added EPS of mushrooms at 500 µg/well concentrations. The cells were cultured for 24 hours at 37°C, 5% CO_2. To the positive and negative controls were used gallic acid (10%) and PBS, respectively. The viability of macrophages was checked by MTT assay method. All tests were carried out in triplicate.
Cell viability of macrophages was calculated:

$$Cell\ viability\ (\%) = [(B - A)/B]\ x\ 100.$$

Where *A* is the average absorbance at 550 nm of treated groups and *B* is the average absorbance at 550 nm of untreated group (control group). Ethic committee, register: n° 213 and process: 23075.013165/2007-58.

2.6 Monosaccharide composition

Approximately 1 mg of EPS was treated with 0.5 ml of trifluoracetic acid (TFA), 1 M, for 1 hour and 120°C. The acid was eliminated by evaporation until dissected, followed by $NaBH_4$ reduction, acetylation and analyze by Gas chromatography (CG) (Lima *et al*., 2007).

2.7 Statistical analysis

Data were expressed as means ± SD and were analyzed statistically with ANOVA. The level of significance was at a *P* value less than 0.01. Data in all the bioassays were statistically evaluated by Tukey's test by variance analysis and *P < 0.05* was considered significant.

3 Results and Discussion

3.1 Kinetics of growth of synergic fermentation of mushrooms

The growth of mushroom as well as quantitative and qualitative yield of the desired product depends on the utilization of nutrients and physicochemical environment in the medium. The production of exopolysaccharide, mycelia, residual sugar, and pH changes were analyzed daily. The submerged fermentation values are the mean of triplicate independent experiments and calculated the standard deviation.

Each synergic fermentation showed different results in pH, biomass, consume of glucose and EPS production during the kinetic. The change in pH of the medium should be taken into consideration as it greatly influences mushroom growth. Co-cultivation Gf + Cs consumed 71.3% ± 0.4 of glucose at 10th day and pH around 3.6 at 8-10 day of fermentation. After 6 days of fermentation, Gl + Cs presented a pH of 4.0 and 32.8% ± 1.4 of glucose. The co-cultivation Gl + Gf showed pH 4.7 and glucose residual of 10.9 (g/L) ± 0.516 at 3th day of fermentation (Table 2).

The pH change during the entire period was marked by glucose consumption which was used to bioconversion of the sugar for biomass production. Cs + Gf showed higher glucose consumed (73%) and biomass production of 4.4 g dw/L ± 0.212. *G. lucidum* obtained 10.9 g dw/L ± 0.359 of biomass and Gl + Cs was 10.5 g dw/L ± 0.542 (Table 2).

The EPS production by synergic fermentation presented the results: 1.09 g/L ± 0.803 (Gl + Cs) at 4 day; 1.23 g/L ± 0.447 (Gl + Gf) at 7 day and 1.03 ± 0.047 (Cs + Gf) at 7 day. Wherever, the results were lower than *G. lucidum* EPS production, 1.82 ± 0.08 at 7th day (Table 2).

Table 2: Values obtained of submerged fermentation of mushrooms during 10 days

Mushrooms	Glucose consumption (%)	pH (min.)	Biomass (g/L)	EPS (g/L)	EPS yield/day
A.brasiliensis	58.0 ± 1.02	4.5 ± 0.02	8.65 ± 0.09	1.69 ± 0.02	0.28
C. sinensis	50.5 ± 3.4	3.7 ± 0.07	9.68 ± 0,5	1.01 ± 0.02	0.20
G. lucidum	35.5 ± 1.5	4.1 ± 0.02	10.04 ± 0.35	1.82 ± 0.08	0.26
G. frondosa	29.5 ± 3.5	5.4 ± 0.11	2.6 ±0,03	1.07 ± 0.02	0.17
Co-cultivation					
Gl+Cs	32.8 ± 1.4	3.9 ± 0.04	10.7 ± 0.13	1.09 ± 0.08	0.27
Gl+Gf	46.0 ± 1.8	4.7 ± 0.04	5.5 ± 0.04	1.23 ± 0.04	0.17
Gf+Cs	71.3 ± 0.4	4.7 ± 0.03	4.2 ± 0.2	1.03 ± 0.04	0.14

Huang and Liu, 2007, studied *Grifola umbrellata* optimization of culture conditions for EPS produced and the initial pH value of 5 was the most efficient.

Papinutti (2010) studied the optimization growth of *G. lucidum* and obtained by the combined addition of malt extract (10 g/L^{-1}) and glucose (10 g/L^{-1}) and under such conditions peaks of fungal biomass and EPS were 4.32 g/L^{-1} and 2.2 g/L^{-1}, respectively. Yang, Yang and Cheng (2009) tested a new method to increase biomass production of *G. lucidum*. When the agitation rate of the bioreactor was maintained at 100 rpm, the mycelium concentration of 1.31 g/L was obtained in 5 days. Alternatively, intermittent agitation scheme was employed and proved to be effective for enhancing mycelium production rate, causing the increase of mycelia concentration to 3.00 g/L.

Fang, Tong and Zhang (2002), *G. lucidum* reached 15,7 g dw/L of biomass production. Leung *et al.* (2009) obtained biomass of *C. sinensis* most rapidly increased between day 1–4 with 21.0 g dw/L, using 40g of glucose. *C. militaris* showed 21.8 g dw/L of biomass production with 70 g/L of glucose (Mao *et al.*, 2005).

Hsieh *et al.* (2006) showed *G. frondosa* cell growth yielded relatively high mycelial biomass 11.22g dw/L ± 1.14 and the maximum EPS production was 2.248 ± 0.107 g/L that was achieved in 4% glucose medium with 0.5% soybean oil.

Tang and Zhong (2002) tested *G. lucidum* EPS production in stirred biorectors and shake flasks, and the results reached 0.87 ± 0.05 and 0.75 ± 0.05 g/L, respectively.

Grifola frondosa showed production yield of exo-polymer of 1.326 g/L, according Cui *et al.*, 2006. The *Grifola umbellata* culture with skim milk as nitrogen source displayed a much higher specific EPS yield of 112.35 mg/g.

Studies demonstrated more concentration carbon source, increased EPS production. Intrinsec and extrinsec factors are important for biomolecules production for each specie and/or for co-cultive.

3.2 Biological Assays

3.2.1 Anti-proliferative activity of EPS on Ehrlich tumor and Sarcoma 180 cells

Exopolysaccharide by mushroom is a well-known biological response modifier (BRM) widely distributed in nature and testes for anticancer activity with limited success due to toxicity.

Exopolysaccharides from mushroom were prepared from synergic fermentation with *C. sinensis*, *G. lucidum* and *Grifola frondosa* species.

The *in vitro* anti-proliferative activity of EPS was tested on two different cancer cell lines, Ehrlich tumor and Sarcoma 180 cells.

The EPS Gl + Gf and Gl + Cs showed higher anti-proliferative activity on Ehrlich tumor cell with 83.29% ± 3,53 at 500 (μg/well) and 60,6% ± 0,955 at 10 (μg/well), respectively. EPS of Gl + Gf for treatment with different concentrations on tumor cells, suggest dose-dependence activity. And EPS Gl + Cs do not presented significant difference in concentrations at 10 – 500 μg/well (Figure 5).

The anti-proliferative activity against Sarcoma 180 cells by Gl + Gf was 65.88 % ± 2.4. EPS of Gl + Cs and Cs + Gf not presented results higher than controls EPS produced by isolate species (Figure 6).

The control species presented inhibition of Ehrlich tumor cells of 57.6% ± 2.66 (*C. sinensis*), 54.0% ± 2.53 (*G. lucidum*) and 49.7% ± 1.24 (*G. frondosa*) (Figure 5).

The anti-proliferative activity of EPS control species against Sarcoma 180 cells of 52.4% ± (*C. sinensis*) 47.46% ± 2.66 (*G. lucidum*) and 34.62% ± (*G. frondosa*) (Figures 1 and 2). Data obtained suggests that the inhibition of cancer cell proliferation was a result of direct action. The Cs + Gf co-cultive no presented results higher than control isolate species. On the other hand, the *in vivo* tests anti-proliferative activities of the EPS may be action indirect.
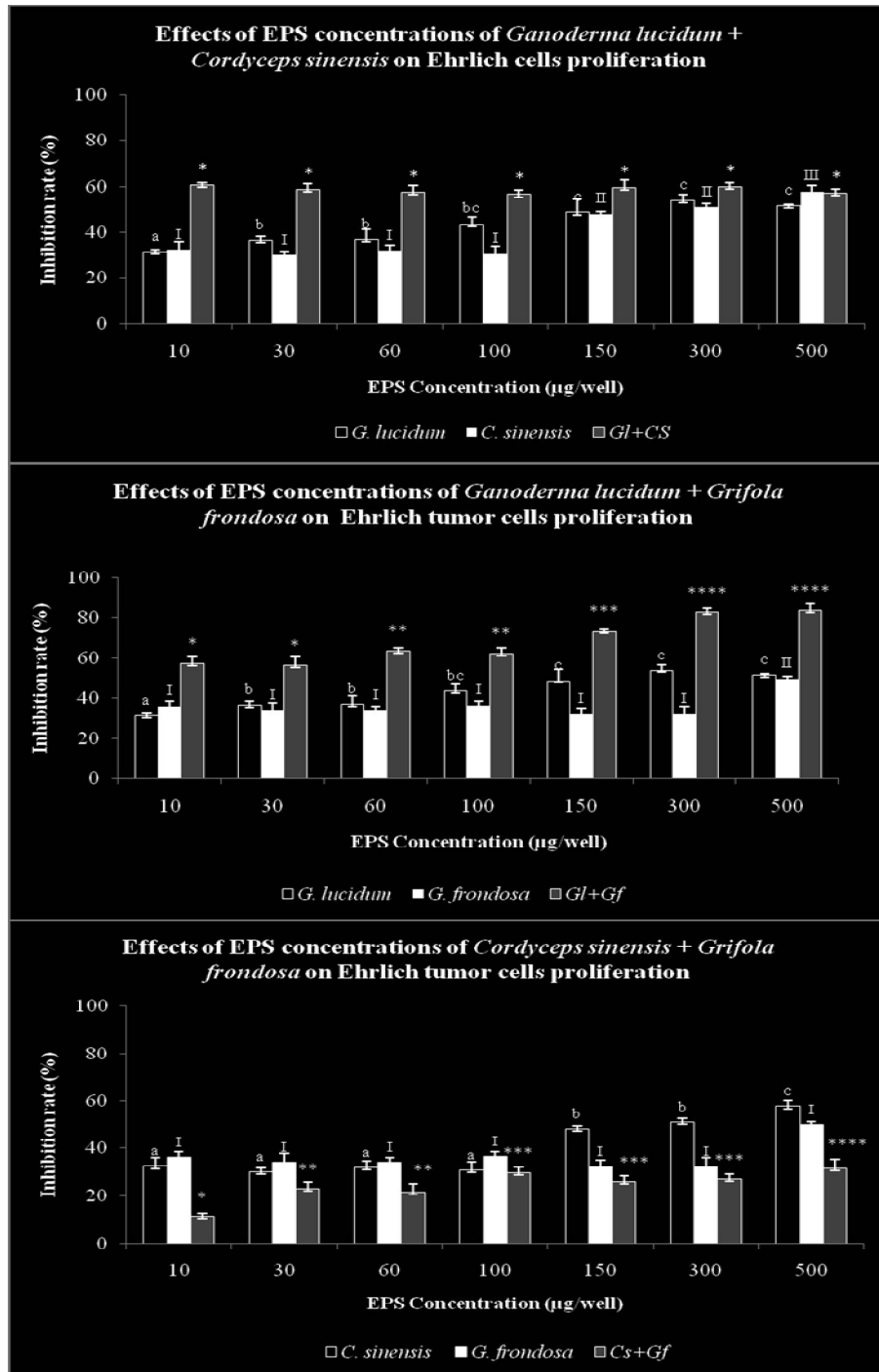
Figure 1: Inhibition of Ehrlich tumor cells proliferation by exopolysaccharides at different concentrations *in vitro*. Each bar represents the mean SD. Different symbols indicate different statistical groups by Tukey´s test at 95% of significance, n =5, *P*<0.05.
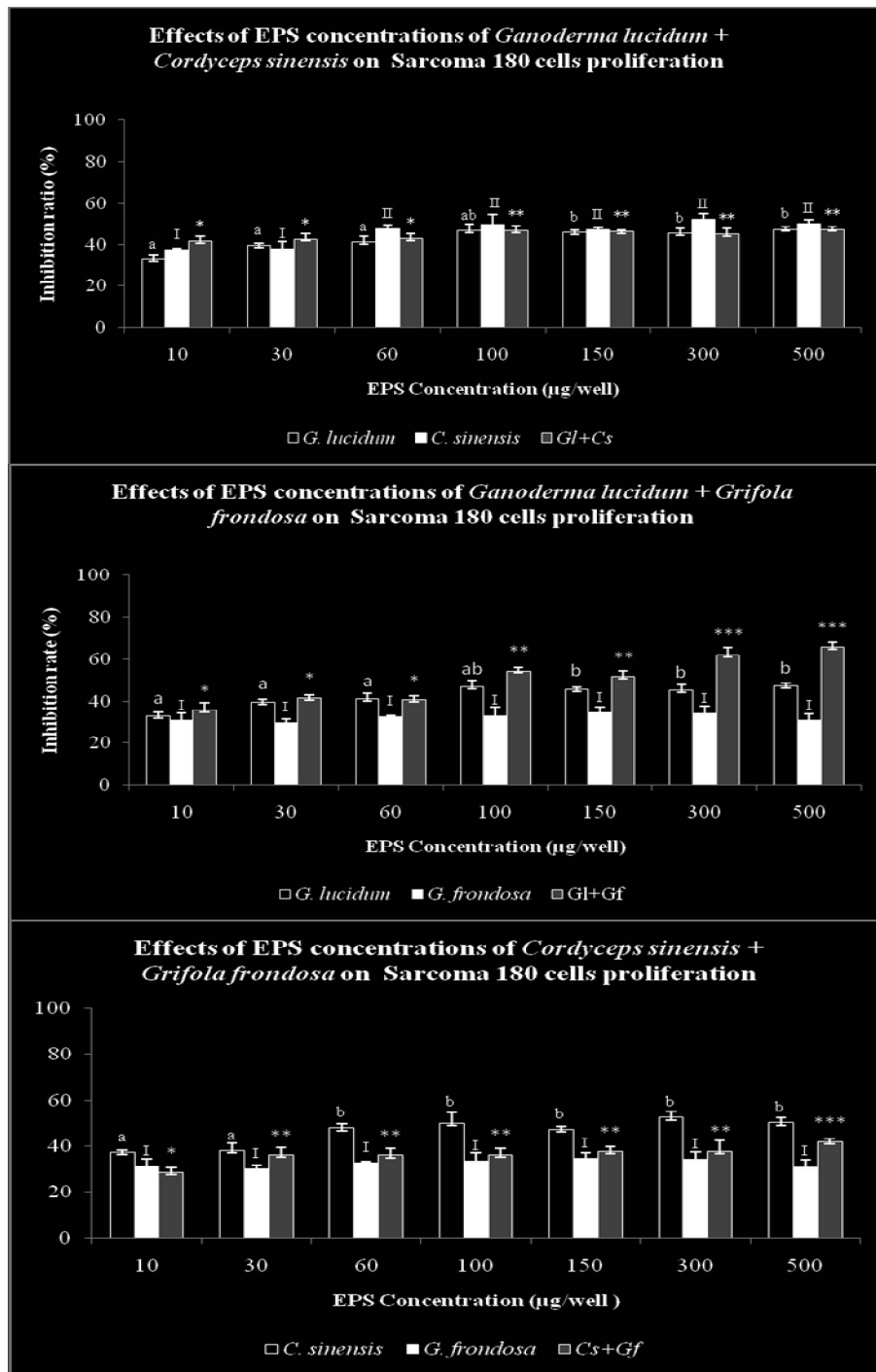
Figure 2: Inhibition of Sarcoma 180 cells proliferation by exopolysaccharides at different concentrations *in vitro*. Each bar represents the mean SD. Different symbols indicate different statistical groups by Tukey´s test at 95% of significance, n =5, *P*<0.05.

G. lucidum showed the highest inhibition rate of MDA cells by GP2-VU-CM was 42.03% of that induced by 25 μg/mL polysaccharide, whereas the rate by GP1-VU-CM was 38.27%, which induced by 50 μg/mL polysaccharide GP-2 (Zhao *et al.*, 2010).

Wu, Zhang and Leung (2005) tested ethanolic extract of *C. sinensis* and obtained significant inhibiting effect on B16-induced melanoma in C57BL/6 mice, causing about 60% decrease of tumor size over 27 days.

The antitumor and immunomodulating activities of the sulfated derivative of EPS of *G. frondosa* were estimated *in vitro* and *in vivo*. EPS inhibited the proliferation of SGC-7901 cells and induced apoptosis, in a dose-dependent manner. And the results from *in vivo* experiments demonstrated that EPS significantly inhibited the tumor growth and enhanced the peritoneal macrophages phagocytosis in S180-bearing mice (Nie *et al.*, 2006).

In vivo tests, the EPS from medicinal mushroom are most likely attributed to effect through multiple mechanisms (immune system, NK cells, macrophages activation, etc) (Wasser, *et al.*, 2002). Studies demonstrated that EPS from mushroom has remarkable immune-enhancing activity. It has been shown to prolong the survival time of radiated mice, stimulate phagocytotic activity of macrophages, and improve the functions of the reticuloendothelial system. With regard to its antitumor properties, it acts directly on tumor cells, as well as indirectly in the host to boost cellular immunity (Zhang *et al.*, 2007).

Studies indicated that cell adhesion is essential step in the initiation of tumor formation: migrating and invasion cell, metastasis and survive in a new environment (Sliva *et al.,* 2002).

Wu *et al.* (2006) studied *G. lucidum* polysaccharides that might affect cancer cell adhesion. Since integrins are the major cell surface adhesion molecules expressed by all cell types. Tests with *G. lucidum* polysaccharides showed the reduction integrin expression. Integrins are composed of α and ß transmembrane subunits. Each α-ß combination has its own binding specificity and signaling properties. Incorporated with various α subunits, ß -integrin binds to diverse extracellular molecules. The fundamental cellular function of integrins is adhesion, and they mediate extensive and important cellular functions by interacting with the extracellular matrix, a process which activates signal transduction.

3.2.2 Cytotoxicity macrophages assay

Macrophages play critical roles in host defense, including phagocytosis of pathogens and apoptotic cell, production of cytokines, and proteolytic processing and presentation of foreign antigens.

The viability of the macrophages treated with EPS produced by synergic fermentation was measured by MTT methods. The control with macrophages, RPMI media plus PBS was considered 100% of viability cells. All treatments presented values higher 90% of cells viability. Gl + Gf obtained 94.41% ± 2.35, Gl + Cs was 91.6% ± 1.23 and Cs + Gf showed 94.08% ± 1.35 of viability cells. The concentration tested was 500 µg/mL.

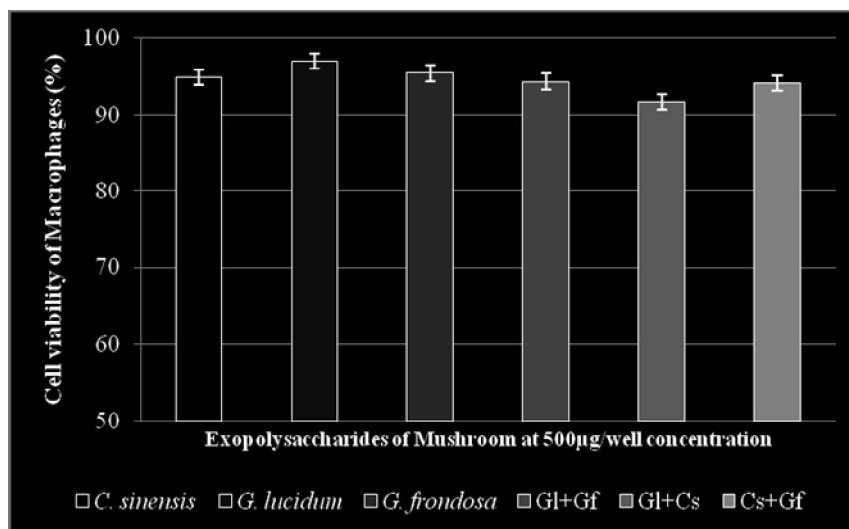

Figure 3: Effect of exopolysaccharides at 500 µg/well concentrations on mice macrophages proliferation *in vitro*. Values are means ± SD of triplicates, n =5, *P<0.05*.

Mushroom polysaccharides prevent oncogenesis, show direct antitumor activity against various allogeneic and syngeneic tumors, and prevent tumor metastasis. Polysaccharides from mushrooms may to attack cancer cells directly and produce their antitumor effects by activating different immune responses in the host. The ß-glucans polymers specifically target macrophages are opsonised with antibody and C3 (complement), and therefore, ß -glucan appears to have the same specificity as opsonising antibody (Wasser *et al*., 2002).

3.3 Monosaccharidic composition

Polysaccharides offer a high capacity for carrying biological information because of their increased potential for structural variability. Therefore, this enormous potential variability in polysaccharide structure gives the necessary flexibility for the precise regulatory mechanisms of various cell-cell interactions in higher organisms.

Composition monosaccharide demonstrated that synergic fermentation caused changes in EPS produced by synergic fermentation. The EPS of Gl + Cs presented glucose (71%) and mannose (18.5%), mainly. The composition of Gl + Gf contain 43.5% of glucose and mannose. Cs + Gf showed composition of 67.5% of mannose and 20.5% of glucose.

The isolated polysaccharide from *C. sinensis*, contains glucose, mannose and galactose in a ratio of 1:0.6:0.75 (Wu *et al.*, 2007). And the total sugar content of *C. militaris* was 92.34%. Its major sugar constituents are mannose (72.22%), galactose (18.61%) and glucose (9.17%) (Jong Seok *et al.*, 2010).

Table 2: Composition monosaccharide of EPS of mushrooms by Gas Chromatographic

Composition monosaccharide (%)					
Mushrooms	Ramnose	Fucose	Ribose	Mannose	Galactose	Glucose
C. sinensis	0.2	0.4	1.6	63.5	6.7	26.7
G. lucidum	-	1.25	1.4	23.6	13.6	58.8
G. frondosa	0.4	0.2	0.38	4.3	0.52	93.7
Gl + Cs	0.61	0.62	0.8	18.5	6.5	71.5
Gl + Gf	0.5	1.7	1.5	43.5	8.0	43.5
Cs + Gf	0.2	4.5	2.3	67.5	5.0	20.5

G. frondosa fruit bodies of this mushroom contain the major component sugar is glucose, while fucose, xylose, mannose and galactose are minor components (Mizuno *et al.*, 1987). It must be stated that the polysaccharide structure in cultured mycelia may depend on the composition of the nutrient medium used for cultivation.

The conformational structural of polysaccharide, molecular weight having plays a dominant role in the antitumor activity.

4 **Conclusions**

Kinetic of submerged fermentation of co-cultivation and respective controls presented important information about EPS and biomass production. The combination Gf + Cs showed high glucose consume. Gl + Cs co-cultivation showed higher production of biomass. However, the lower pH was isolate cultivation of *C. sinensis*. And the isolate cultivation of *G. lucidum* showed better results for production of biomass and EPS.

EPS produced by synergic fermentation shown interesting anti-proliferative properties on different cancer cells and relatively low toxicity to macrophages. These properties show that the EPS of Gf + Gl and Gl + Cs is a potential source of natural anti-proliferative Ehrlich tumor cells. And Sarcoma 180 cells were inhibited by isolated cultures around 50% cell proliferation, therefore, the co-cultivation no present results higher than controls.

In macrophages viability tests, all samples no showed cytotoxic effect in macrophages cells. Composition monosaccharide was different for all samples. Co-cultivation method produced new compositions for each EPS.

The importance this study is that we obtained new compounds by co-cultivation of mushrooms and anti-proliferative activity on tumor cells. This research offers a new perspective for prevention and/or treatment of cancer.

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