UNIVERSIDADE FEDERAL DO PARANÁ

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ANTILEISHMANIAL BIOCOMPOUNDS SCREENING ON SUBMERGED MYCELIAL CULTURE BROTHS OF TWELVE MACROMYCETE SPECIES

CURITIBA
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Dissertation presented as a partial requisite for the obtention of a master's degree in Bioprocesses Engineering and Biotechnology from the Bioprocesses Engineering and Biotechnology post-Graduation Program, Technology Sector, Federal University of Parana.

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Prof. Dr. Vanete Thomaz Soccol

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RESUMO

Muitas parasitoses continuam representando sérios problemas de saúde por todo o mundo. As leishmanioses, que são algumas das mais devastadoras doenças conhecidas, estão reemergindo e se difundindo. Os agentes quimioterápicos para tratar leishmanioses não são satisfatórios e os parasitas continuam a evoluir. Os experimentos descritos na presente dissertação são parte de um esforço no sentido de desenvolver melhores agentes quimioterápicos para tratar leishmanioses. Sessenta extratos, obtidos de 12 espécies distintas de macromicetos foram prospectadas para atividade anti-leishmânia contra três espécies do gênero *Leishmania*. Todas as cepas de cogumelos testadas foram especialmente coletadas para este experimento. Procedimentos extrativos foram aplicados aos caldos resultantes de cultivos miceliais. Processos extrativos foram selecionados objetivando a purificação das classes mais promissoras conhecidas de moléculas com atividade anti-leishmânia: alcaloides, quinonas e terpenos. Foram utilizadas metodologias envolvendo timidina radiomarcada e MTT para avaliar a bioatividade anti-leishmânia dos extratos. Os resultados foram estatisticamente analisados por ANOVA e as diferenças entre os tratamentos e grupos controle foram determinadas por pós-teste de Dunnett, para 1% e 5% de significância ($P<0,01$ and $P<0,05$). Atividades expressivas foram encontradas em pelo menos dezenove dos extratos, mesmo em relação ao Glucantime 300mg/mL. Quatro dos extratos apresentaram mais atividade que o controle positivo. Três contra *L. enrietti*, três contra *L. braziliensis* e duas contra *L. infantum*. Dezenove extratos se mostraram tão ativos quanto o controle positivo, nove dos quais contra *L. infantum*, dezesseis contra *L. enrietti* e dez contra *L. braziliensis*. As espécies mais promissoras identificadas por este estudo foram *Plectania* sp*., Ganoderma stipitatum, Oudemansiella canarii* e *Perenniporia martiusii*. Os extratos brutos e preparações de quinonas foram os mais ativos. O concentrado bruto obtido do cultivo de *Plectania* sp. foi ativo contra as três espécies testadas de leishmânias. *Oudemansiella canarii* é reconhecidamente comestível, podendo apresentar potencial para desenvolvimento de nutracêuticos. Os produtos mais ativos indicados pelos experimentos aqui descritos devem ser avaliados em outros experimentos *in vitro* e *in vivo*, incluindo testes clínicos, para comprovar a eficácia das substâncias, bem como a ausência de efeitos colaterais. Os experimentos descritos tiveram êxito em avaliar um grande número de amostras em um curto período. As metodologias utilizadas são suficientemente rápidas, práticas e sensíveis para a seleção de compostos com atividade anti-leishmânia para estudos subsequentes.

Palavras-chave: cogumelos, macromicetos, leishmaniose, screening, fármacos, MTT, timidina radiomarcada

ABSTRACT

Many parasitoses persist as serious health problems all over the world. Leishmaniases, wich are some of the most devasting diseases known, are constantly reemerging and diffusing. Chemoterapeutic agents for treating leishmaniases are not satisfactory and parasites continue to evolve. The experiments described in this dissertation are part of an effort to develop better chemotherapeutic agents to treat leishmaniases. Sixty mushroom extracts, obtained from 12 distinct macromycete species were screened for antileishmanial activity against three species from *Leishmania* genus. All of the tested mushroom strains were specially isolated for this experiment. Extractive procedures were applied to the liquid broths obtained by the submerged cultivation of the macromycetes mycelia. Extractive procedures were designed to obtain the most promisor chemical classes of molecules with antiparasitic activity known: alkaloids, quinones and terpenes. MTT and radiolabelled thymidine methodologies were applied for antileishmanial bioactivity evaluation of the extracts. Results were statistically analysed by ANOVA and differences between the treatment and control groups were determined by Dunnett´s post test for 1% and 5% levels of significance ($P<0,01$ and $P<0,05$). Expressive activities were found on at least nineteen extracts, even in comparison to Glucantime 300mg/mL. Four extracts had shown even more activity than the positive control. Three against *L. enriettii,* three against *L. braziliensis* and two against *L. infantum*. Nineteen extracts were found to be as active as the positive control. Nine of wich against *L. infantum,* sixteen against *L. enriettii* and ten against *L. braziliensis*. The most promisor mushroom species suggested by this experiment are *Plectania* sp*., Ganoderma stipitatum, Oudemansiella canarii* and *Perenniporia martiusii*. Gross concentrated and quinones preparations were the most active ones. *Plectania* sp. concentrated extract was more active than the positive control for all three leishmania species assayed. *Oudemansiella canarii* is edible, having potential for the development of nutraceuticals. Compounds that showed a high antiparasitic activity in this study should be further tested by other complementar *in vitro* methodologies, and even through *in vivo* assessments and clinical trials to comprove the activities and check the absence of side effects. The experiments were successful in assessing a great number of preparations for their antileishmanial activity. The adopted methodologies are sufficiently fast, practical and accurate, to point potential novel antiparasitic substances for further studies.

Keywords: mushrooms, macromycetes, leishmaniosis, screening, pharmaceuticals, MTT, radiolabelled thymidine.

FIGURES LIST

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ACRONYMS LIST

AU	– *Auricularia fuscosuccinea*
G3	– *Ganoderma australe*
GA	– *Ganoderma applanatum*
GS	– *Ganoderma stipitatum*
LM	– *Lycoperdon marginatum*
LS	– *Lepista sordida*
OC	– *Oudemansiella canarii*
PC	– *Pleurotus calvescens*
PD	– *Pleurotus djamor*
PM	– *Perenniporia martiusii*
PL	– *Plectania* sp.
PS	– *Pycnoporus sanguineus*
WRF	– white rot fungus
CL	– cutaneous leishmaniasis
MCL	– mucocutaneous leishmaniasis
DCL	– diffuse cutaneous leishmaniasis
VL	– visceral leishmaniasis
PKDL	– post kala-azar dermal leishmaniasis
SCL	– sporadic cutaneous leishmaniasis
BrdU	– Bromodeoxyuridine
MTT	– 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium
SAG	– sodium antimony gluconate
SDS	– sodium dodecyl sulphate
WST-1	– (4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolium]-1,3-benzene disulfonate)
XTT	– (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)
DNA	– deoxyribonucleic acid
rDNA	– ribossomic deoxyribonucleic acid
BHI	– brain heart infusion
CCS	– millieu Coeur Cerveaux sang

FCS	– fetal calf serum
LIT	– liver infusion tryptose
MEA	– malt extract agar
NNN	– Novy-MacNeal-Nicolle
PBS	– phosphate buffered saline
PDA	– potatoe dextrose agar
RPMI	– Roswell Park Memorial Institute
PCR	– Polymerase Chain Reaction
RAPD	– Random Amplification of Polymorphic DNA
RFLP	– Restriction Fragment Length Polymorphism
HDL	– high density lipoproteins
LDL	– low density lipoproteins
ANOVA	– analysis of variancy
CPM	– counts per minute
EMBRAPA	– Empresa Brasileira de Pesquisa Agropequária
IBMP	– Instituto de Biologia Molecular do Paraná
PR-Metrologia	– Rede Paranaense de Metrologia e Ensaios
SENAI	– Serviço Nacional de Aprendizagem Industrial
SPVS	– Sociedade de Pesquisa em Vida Selvagem
UFPR	– Universidade Federal do Paraná
WHO	– World Health Organization

CONTENTS

1 INTRODUCTION

As most of the life forms over the earth show some parasitic behaviour, it is not surprising that parasitoses have always been a challenge for humanity. We are now technically able to prevent or cure many infectious diseases and parasitoses. Infectious diseases, once the main cause of death in the world are killing less people today than cancer or cardiovascular diseases (DE PUYORAC, 2001).

However, although medicine, associated with pharmacology had progressively evolved, many parasitic diseases persist as a serious health problem all over the world (BALCIOGLU, 2007; CASTRO *et al*., 2005).

Researchers are in a constant pursuit for natural compounds with antiparasitic activity. Plants, fungi and even animals are being tested as sources of antiparasitic substances. Although many of these compounds have shown some degree of activity, the most aggressive ones also seem to damage human cells, being not therapeutically useful (KAYSER, KIDERLEN & CROFT, 2002; SINGH & SIVAKUMAR, 2004).

Macromycetes, popularly called mushrooms, are a source of great molecular diversity. This, besides the fact almost 95% of fungi are unknown, makes them interesting as a source of novel organic structures (PACCOLAMEIRELES, 2002; SOCCOL *et al*., 2006; MORADALI *et al*., 2007).

Although there are already some evidences of antiparasitic properties in several fungi molecules, the number of mushroom species and molecules that still untested is astonishing, specially in a biologically rich country as Brazil.

Experiments here presented were designed to evaluate the antiparasitic activity of mushrooms derived biomolecules. The bioactivities of twelve mushrooms species (sixty different mushrooms extracts) were evaluated against three different species of the genus *Leishmania* (*L. braziliensis, L. enriettii* and *L. infantum*), through two distinct methodologies (MTT and radiolabelled thymidine).

With a screening character, this experiment is intended to be the first step on a therapeutic substances discovering/development process. This also should contribute to methodology adequation and consolidation, as there are virtually infinite more biomolecules to assess in future experiments.

2 REVIEW

2.1 PARASITES

Parasitism is the relationship between two species of organisms in which one benefits at the expense of the other, sometimes without killing it (BRITANNICA, 2007). This is probably the most successful survival strategy adopted in nature. It is estimated that the majority of all living species of organisms are parasites (WINDSOR, 1998).

This experiment focused leishmaniases for being serious diseases, that cause the suffering and death of millions of humans and other mammals, affecting many countries, including Brazil and that still need better therapies (STROM, 2006; SINGH & SIVAKUMAR, 2004; KAYSER, KIDERLEN & CROFT, 2002; NOLETO *et al*., 2002).

2.1.1 Leishmaniases

Leishmania spp. (FIGURE 1) are protozoaries (hemoflagellates) that cause a group of chronic diseases, known as leishmaniases, which affect humans, as well as many other mammals. Leishmaniases are among the six major parasitic diseases groups targeted by World Health Organization (GOMES *et al*., 2008).

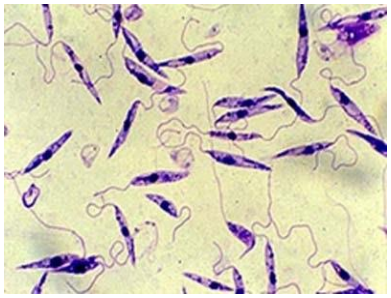


FIGURE 1 – LEISHMANIAS IN THEIR FLAGELLATED FORM. SOURCE: FIOCRUZ (2008).

People affected by those protozoaries are spread among at least 88 countries in the world (72 of wich are developing countries). Leishmaniases are prevalent in the northern Africa, Asia, Mediterran and Latin America, from southern Mexico to northern Argentina (WONG & CHOW, 2006; THOMAZ-SOCCOL *et al*., 2000).

Leishmaniases can manifest itself in one of the following four most common forms, depending on several factors, including the causative species and immunological condition of the human host:

- cutaneous leishmaniasis (CL),
- diffuse cutaneous leishmaniasis (DCL),
- mucocutaneous leishmaniasis (MCL),
- visceral leishmaniasis (VL).

Cutaneous leishmaniasis (CL) is recognised as a complex and highly variable disease in terms of its epidemiology, aetiology, pathology and clinical presentation. It is mainly characterized by epithelial lesions that evolve and get suceptible to secondary infections (FIGURE 2). Lesions may simulate other disease conditions, as tumour-like lesions and, when ulcerated, may resemble basal cell carcinomas, especially in older individuals, making diagnosis difficult, particularly in nonendemic regions (CAUMES & BOURÉE, 2008; KUMAR *et al.*, 2008).

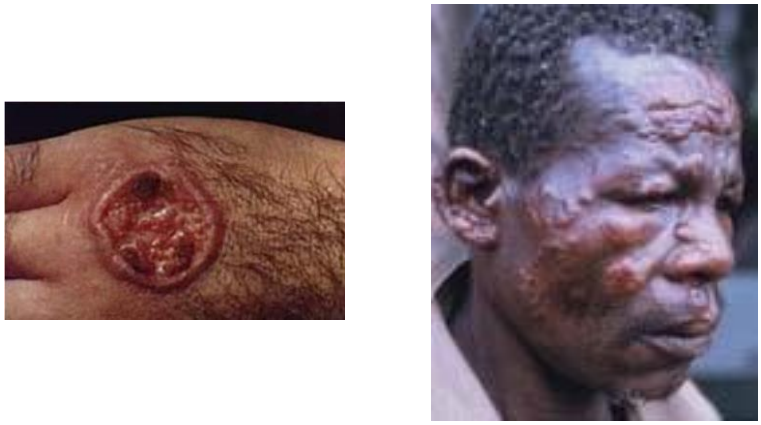


FIGURE 2 - CUTANEOUS (LEFT) AND DIFFUSE-CUTANEOUS (RIGHT) LEISHMANIASES.
SOURCES: Thomaz-Soccol (2005) AND WHO (2008b).

Diffuse cutaneous leishmaniasis (DCL) is charachterized by disseminated and chronic skin lesions, similar to lepromatous leprosy (WHO, 2008). It is an extremely severe clinical form of tegumentary leishmaniasis. Disseminated lesions can cover much of the body, for the whole life, resulting in social and psychological problems (AZEREDO-COUTINHO *et al*., 2007).

Mucocutaneous leishmaniasis (MCL) is usually caused by New World species of *Leishmania*, but has occasionally occurred following infection with other species.

Clinically, the lesions present either as long-lasting ulcers on the tongue, palate, labial commissure or lip, or as a diffuse nodular swelling of the gums and palate (FIGURE 3). The disease course usually takes about 12 months from onset of symptoms to diagnosis, because the symptoms are usually mistaken for neoplasia (ANTINORI *et al*., 2008).

Visceral leishmaniasis (VL), popularly called Kala-Azar or black fever, is the most severe form of leishmaniasis. It is the second most fatal parasitosis in the world, just after malaria. About 500.000 people get infected and 60.000 die from VL every year in the world. Parasites migrate to the liver, spleen and bone marrow (PINHEIRO *et al*., 2008).

Symptoms include: fever, blood cells abnormalities, splenomegaly (augmented spleen) and sometimes hepatomegaly (FIGURE 3). Darkening of skin is caused by some *L. donovani* strains. VL is caused by several leishmania species, including *L. infantum*, *L. donovani* and *L. chagasi*. Those three species are known as *L. donovani* complex. Those protozoa had been reported in many countries, including China, India, mediterranean countries, african and south american countries (ANTINORI *et al*., 2008).

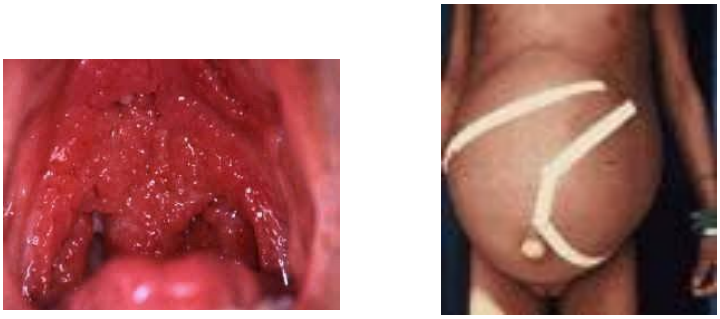


FIGURE 3 - MUCOCUTANEOUS LEISHMANIASIS ULCERATION (LEFT) AND VISCERAL LEISHMANIASIS (RIGHT). SOURCES: Thomaz-Soccol (2005) AND WHO (2008b).

Without proper treatment, VL cases usually evolve to fatal endings. Although death can be caused by the parasite itself, some parallel pathologies, as pneumonia, tuberculosis and dysentery can contribute or determine the obit. Progress of the disease can take one to 20 weeks. If untreated for two years, it´s fatality rate is 100% (WHO, 2008). But even when the patient recovers, there is a possibility the patient develops, after one month to even years, a pathology known as post kala-azar dermal leishmaniasis (PKDL). This condition is characterized by the appearing of many small lesions on the face that spread and evolve to the point of disfiguring and blinding the affected individual.

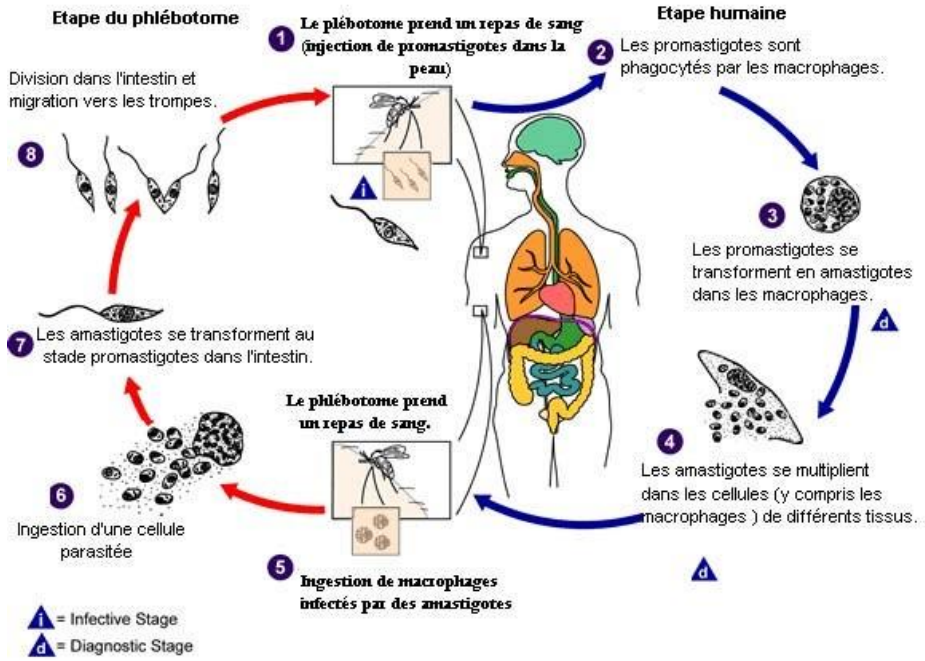


FIGURE 4 - LEISHMANIAS LIFE CYCLE. SOURCE: CDC (2008).

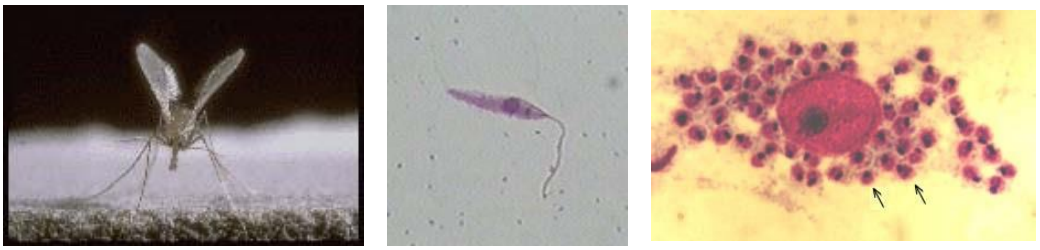


FIGURE 5 - PHLEBOTOMINE SANDFLY (LEFT). SOURCE: WHO (2008b). PROMASTIGOTE (MIDDLE) AND AMASTIGOTE (RIGHT) FORMS OF *Leishmania* sp. SOURCE: UFRGS (2008).

Leishmanias life cycle (FIGURE 4) has two distinct phases: one inside insect vectors and other parasiting mammals (FIGURE 5). The parasites can be transmitted through the bite of infected female phlebotomine sandflies. When mosquitoes have bloodmeals on infected mammals, they become infected by leishmania amastigotes, that come within infected macrophages. On insects intestines, amastigotes are released from macrophage cells and transform into promastigotes. When the mosquito have another bloodmeal, it can infect another mammal (ECCO *et al*.; 2000).

Many animal species, including the genera *Akodon, Oryzomys, Nectomys* and *Bolomys* were identified as reservoirs of leshmanias, suggesting the existence of a wild cycle. No horses were find to be infected. Dogs, once thougt to be the primary reservatory

of the disease, didn´t show high rates of infection for the evaluated areas (CASTRO *et al*., 2005; CASTRO *et al*., 2007). Dogs, as humans, seem to be accidental hosts (FIGURE6). Most of the human and canine infections occur in forest or forest-edge areas. Most of the people are infected when entering the forest, for extractive, touristic or scientific purposes (CASTRO *et al*., 2005).

In the mammalian host *Leishmania* spp. are obligate intracellular parasites, wich infect haematopoietic cells of the monocyte/macrophage lineage. Parasites enter cells by phagocytosis, and subvert cellular defence systems, as nitric oxide and cytokine mediated mechanisms, proliferating and affecting the immunological condition of the host (ANTINORI *et al*., 2008).

FIGURE 6 **-** CANINE MUCOCUTANEOUS LEISHMANIASIS. SOURCE: Thomaz-Soccol (2005).

The *Leishmania* Ross, 1903 genus belongs to the Kinetoplastida order, Trypanosomatidae family. Species of the *Leishmania* genus have been classified into two subgenera based on extrinsic eco-epidemiological criteria and Linnean taxonomy: *Leishmania* Saf'Janova, 1985 and *Viannia* Lainson & Shaw, 1987. More than 17 taxa that have been described in the New World were regrouped into phylogenetic complexes based on biochemical techniques and phenetic/cladistic approaches (THOMAZ-SOCCOL, 1993). Four phylogenetic complexes were identified by isoenzyme electrophoresis: *L. infantum, L. braziliensis, L. amazonensis* and *L. guyanensis/panamensis* (THOMAZ-SOCCOL *et al*., 2000).

Latin-American *Leishmania* species belong to both taxonomic subgenera:

- The subgenus *Leishmania*, composed of *Leishmania* (L.) *mexicana* and *Leishmania* (L.) *amazonensis*, responsible for localized or diffuse cutaneous

disease; and *Leishmania* (L.) *infantum chagasi*, the cause of New World viscerotropic leishmaniasis.

- The subgenus *Viannia,* wich causes New World cutaneous leishmaniasis with cutaneous or mucocutaneous lesions. It comprises the species *Leishmania* (V.) *braziliensis*, *L.* (V.) *panamensis*, *L.* (V.) *guyanesis* and others.

Although having similar clinical appearance, infection by different species of *Leishmania* have distinct prognostics. Ulcers caused by *Leishmania* spp*.* of the subgenus *Viannia* are more aggressive and can recur after treatment. The ulcers caused by parasites of subgenus *Leishmania* are less severe and more likely to cure spontaneously or after treatment. On the other hand, only species from the *Leishmania* subgenus cause the visceral form of the disease, wich is the most serious and fatal.

Diagnosis in regions that are endemic both for the cutaneous and visceral forms (as Sao Paulo state, in Brazil for example) must be very accurate, for the proper medical tratment and drugs prescription. Molecular techniques (based in PCR and/or enzymes electrophoresis profiles) for differential leishmaniases diagnostic have been developed and are currently being used (GOMES *et al*., 2008; CASTRO *et al*., 2005; THOMAZ-SOCCOL, 1993). The first clinical descriptions of cutaneous leishmaniasis were made in 1756 by Alexander Russel. But some Inca potteries, found on Ecuador and Peru, dating back to the first century AD, have representations of skin lesions and facial deformities. It is believed that cutaneous and mucocutaneous leishmaniasis prevailed in the New World as early as this (WHO, 2008b).

Leishmaniases are reemerging and spreading because of many risk factors: essentially humanmade and environmental changes, the immune status of the host and drug resistance (SERIDI *et al*. 2008).

Global temperature rising and alterations on pluviometric indexes can propiciate a rise in the populations of vectors and reservoirs. Those environmental changes also alterate the behaviour of animals: mosquitoes accelerate their metabolism, reproducing faster and having more frequent bloodmeals to avoid dehydration in hot climates. They also move to previously unaffected areas in search for better climates and sources of food, generating new focuses of infectious diseases. Rodents move to residential areas when their habitat is flooded or excessively dry (VORMITTAG, 2008). Recently there was

a leishmaniasis epidemy on Mato Grosso do Sul state, in Brazil and Rio de Janeiro state is now facing a serious epidemy of dengue, wich is another disease transmitted by mosquitoes. Both diseases had almost disapeared for several decades and are now reemerging with more strenght.

More than 90% of the cutaneous cases appear in Afghanistan, Saudi Arabia, Algeria, Brazil, Iran, Iraq, Syria, and Sudan, whereas more than 90% of visceral cases appear in India and Sudan (FIGURE 7). The mucocutaneous form is mostly found in Latin America (SINGH & SIVAKUMAR, 2004).

Organ transplantation and blood transfusion were recently found as means of infection for all leishmaniases forms, especially visceral. Infection can process directly through a contaminated organ or blood, or can be previously present and only activated by the immunossupressive treatment, necessary for transplantation acceptance. In both cases, immunosupression contributes to disease progression (ANTINORI *et al*., 2008).

It is estimated that there are approximately 500.000 new cases of visceral forms and 1,5 million cases of cutaneous forms each year over the world. In Brazil there are approximately 4.000 new cases of visceral forms and 33.000 new cases of cutaneous forms each year (GOMES *et al*., 2008). In the state of Parana (FIGURE 8), southern Brazil, 3906 human cutaneous leishmaniasis cases were recorded between 2000 and 2005 (CASTRO *et al*, 2007). According to the World Health Organization (2008b), 350 million people suffer from leishmaniasis in the world, nowadays.

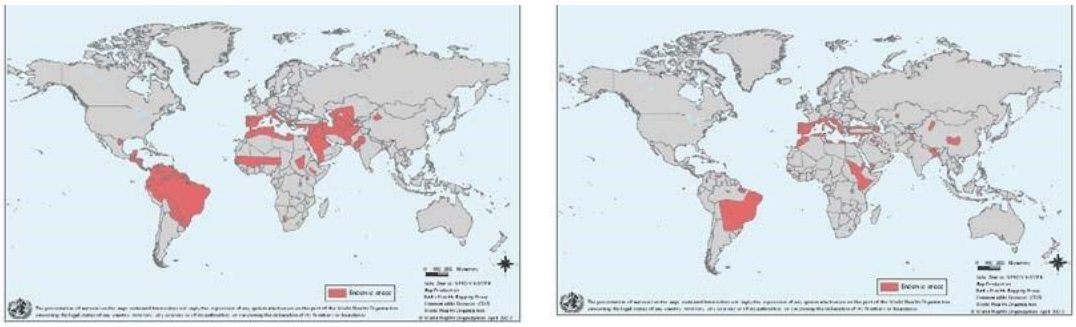


FIGURE 7 **-** CUTANEOUS (LEFT) AND VISCERAL (RIGHT) LEISHMANIASIS WORLD DISTRIBUTION. SOURCE: WHO (2008c).

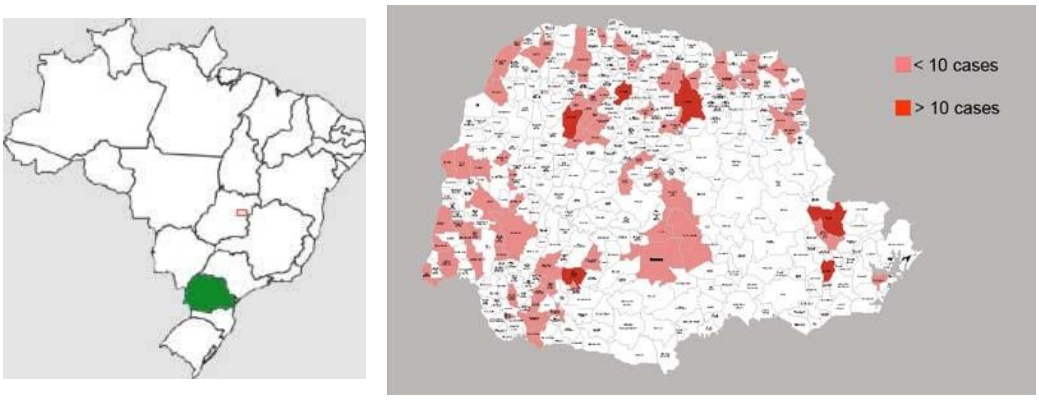


FIGURE 8 - BRAZIL MAP, WITH HIGHLITED PARANA STATE (LEFT). SOURCE: Hostels (2008). CUTANEOUS LEISHMANIASIS DISTRIBUTION IN PARANA STATE, BRAZIL (RIGHT). SOURCE: Thomaz-Soccol (2005).

There follows a brief review about the three *Leishmania* species tested for the present experiment: *L. braziliensis,L. enriettii* and *L. infantum.*

2.1.1.1 *Leishmania* (Viannia) *braziliensis*

L. braziliensis is the main ethiological agent of human tegumentary leishmaniasis in South America. It is widely distributed and adapted to many different sandfly vectors and reservoir hosts. Vectors include the sandfly species *Lutzomyia youngi*, *L. columbiana* and *L. whitmani.*

In Brazil, rodents are believed to be the main animal reservatory for *L. braziliensis*. Rodent species include those of the genera *Akodon, Oryzomys, Proechimys, Rattus and Rhipidomys* (ALEXANDER *et al*., 1998; BRANDÃO-FILHO *et al*., 1999, 2003). Dogs, once thougt as the main reservoirs of leishmaniasis, were found to have low rates of infection by the protozoary (CASTRO *et al*., 2007).

Almost all serious mucocutaneous leishmaniasis cases of the Americas are associated with *L.*(V.) *braziliensis* (BRANDÃO-FILHO *et al*., 1999). In Brazil, cutaneous leishmaniasis is caused by six *Leishmania* species, but the vast majority of cutaneous lesions are caused by *L.* (V.) *braziliensis* (GOMES *et al*., 2008; CASTRO *et al*., 2005).

In other regions of the world, as mediterranean countries, cutaneous leishmaniasis is mainly caused by *Leishmania tropica* (PARVIZI *et al*., 2008). In Colombia, tegumentary leishmaniasis is mainly (more than 85% of the cases) caused by

species from the *L. guyanensis/panamensis* complex (THOMAZ-SOCCOL *et al*., 2000). In Texas, USA, *L. mexicana* is the only cutaneous leishmaniasis ethiologic agent.

Differential diagnostic is necessary for regions with incidence of both *L. braziliensis* and *L. mexicana*, as *L. braziliensis* needs aggressive treatment (WRIGHT *et al*., 2008). The main region of cutaneous leishmaniasis incidence in Brazil is the northeast of the country, but almost all states are affected. Almost all Latin America have the incidence of this parasite. Exceptions are Chile and Uruguay (VIDIGAL *et al*., 2008).

In the state of Paraná, southern Brazil, 3.906 human cases of tegumentary leishmaniasis were recorded between 2000 and 2005. *L. braziliensis* is the only leishmania species present in this region and *Lutzomyia. whitmani* was found to be the main vector (CASTRO *et al*., 2007).

2.1.1.2 *Leishmania* (Leishmania) *enriettii*

This parasite species pertains to *L. amazonensis* phylogenetic complex, wich is associated with diffuse cutaneous manifestation of leishmaniasis (DCL). In fact, the diffuse cutaneous form is the most frequent manifestation of infections by this phylogenetic group, but the cutaneous, mucocutaneous and even visceral forms are also reported, depending on a number of factors, including the immunological status of the host (CARVALHO *et al*., 2008).

Diffuse cutaneous leishmaniasis (DCL) is characterized by an absence of cellular immunulogical response and a great number of amastigotes inside macrophages. The infection progress is usually difficult to control and available therapies not efficient (AZEREDO-COUTINHO *et al*., 2007).

In Brazil, the only species associated with human DCL is *L. amazonensis* (AZEREDO-COUTINHO *et al*., 2007). But, *L. enriettii* was found as a natural parasite of guinea pigs in this country. Its relation with the host is similar to that of *L. braziliensis* (OMD, 2008). It also parasites other mammals, as hamsters. However, ulcerations and metastatic lesions are not produced in hamsters (BELEHU & TURK, 1976).

As some strains of this species are not reported to be pathogenic to humans, these are used as a living vaccine and as prototype organisms, for drug testing and many

diverse genetic manipulations. Laban *et al*. (1990) have introduced the bacterial neomycin gene on a *L. enriettii* strain.

Other strain of this species was genetically modified to produce a foreign immunogenic protein: circumsporozoite protein (PyCSP) of *Plasmodium yoelii*, aiming the development of an immunization technique against malaria and as a prototype against other infectious diseases (WANG *et al*., 1995).

2.1.1.3 *Leishmania* (Leishmania) *infantum*

L. (L.) *infantum* produces a spectrum of clinical forms, ranging from visceral (VL) to cutaneous (CL) leishmaniasis, affecting humans and other mammals as dogs (FERNÁNDEZ-BELLON, 2008). VL caused by *L. infantum* occurs mainly in infants. This is the ethimological origin of the species name. The sandflies *Phlebotomus perniciosus* and *P. perfiliewi* are the main vectors of VL and CL, respectively, but other *Phlebotomus* species, as *P. ariasi* are also known to transmit those parasitosis (SERIDI *et al*., 2008).

These forms of leishmaniasis are commonly found in Mediterranean countries. Viscerotropic *Leishmania* (L.) *infantum* have been isolated from cutaneous lesions in Iran, Turkey, France, Algeria, Spain, Greece, Italy, Portugal and Tunisia. But only a few strains have been identified. Results have shown that *L. infantum* is responsible both for the visceral and cutaneous forms of the disease (KALLEL *et al*., 2008).

India is one of the world´s largest foci of visceral leishmaniasis (almost 50% of all cases). Since 2002 there is a govenamental program to face this situation, with administration of miltefosine, hoping to erradicate the disease until 2010 (WHO, 2008d). Domestic dogs were found to be the main animal reservoir of *L. infantum* parasites. *L.infantum* also causes canine leishmaniasis (PARVIZI *et al*., 2008).

Sporadic Cutaneous Leishmaniasis (SCL) can also be caused by *L. infantum*. The sporadic form (SCL) is only described in northern Tunisia with approximately 20 or 30 cases per year. Clinically, SCL is characterized, in more than 80% of cases, by a single lesion on the face with the appearance of an ulcerous lesion with scabs. *Phlebotomus perfiliewi* as well as *P. langeroni* are suspected vectors in the transmission of SCL, whereas no animal reservatory has yet been identified (KALLEL *et al*., 2008).

Visceral leishmaniasis can also manifest diffuse cutaneous dissemination in HIV debilited patients (MANFREDI *et al*., 2008).

2.2 ANTIPARASITICS

2.2.1 Antiparasitic biomolecules

Miriads of biomolecules have shown some kind of antiparasitic activity through experimental methods. Some examples are: phenols and phenolic acids, cumarines, nitroimidazoles, quinones, including naphtoquinones, hidroxinaphtoquinones and anthraquinones, polyene ionophores, xanthones, alkaloids, steroids, quinolines, bibenzylisoquinolines, indoles, nucleosides, aminoglucosides, ajoenes, organic acids, lipids, acetogenins, lignans, chalcones, aurones, flavonoids and terpenoids, including iridoids, monoterpenes, sesquiterpene lactones, diterpenes, triterpenes, limonoids, quassinoids among many others (KAYSER, KIDERLEN & CROFT, 2002).

Three among all these classes of bioactive molecules were chosen for this experiment, as they have shown probably the most strong direct *in vitro* cytotoxic effects over parasites: alkaloids, quinones and terpenes.

Following is a brief review about each of these chemical classes.

2.2.1.1 Alkaloids

There is no clear definition for this class of molecules, but all alkaloids are pharmacologically active nitrogenated compounds. There is no difference between some natural complex amines and alkaloids. According to Pelletier (1988), alkaloids are cyclic organic substances that contain a nitrogen atom in negative oxidized state, and have limited distribution among living organisms.

Although not perfect, Pelletier definition is very approximate, because include all of the known alkaloids and exclude many nitrogenated compounds as: simple amines, aminoacids, peptides, proteins, nucleic acids, nucleotides, porfirins, vitamines, nitro and nitrous compounds.

They are predominantly alkaline, but there are exceptions such as colchicine, piperine, oximes and some quaternary salts, as lauripholin. They frequently form salts with organic acids, as quinic or meconic acid. Many of them are optically active, for being assimetric. Some combinate with sugars, as solanin and others occur as esthers or amides, as paclitaxel.

Molecules with a nitrogen atom in a heterocyclic ring are called true alkaloids. Molecules in wich nitrogen doesn´t belong to heterocyclic systems are called protoalkaloids and nitrogenated compounds that aren´t derived from aminoacids are called pseudoalkaloids (HENRIQUES *et al*.; 2003).

Alkaloids molecular diversity is comparable to that of terpenoids. They represent almost 20% of the natural substances yet described. Their functions were ignored for a long time, but now they are known to have essential roles in vegetals. It has been observed that many alkaloid producing plants are avoided by animals and insects, probably for their toxicity or bitter taste. It was also suggested that they act as hormones or growth inhibitors (allelopathic substances). They may also be involved in ionic equilibrium for their alkalinity. Other, less strong hypothesis are that alkaloids are a mean of detoxification or nitrogen reservatories (PELLETIER, 1988).

As well as other secondary metabolites, alkaloids have proven to contribute with plant defenses against parasites (TANAKA *et al*.; 2007). Potatoes, for example, exacerbate solanin production when under microbial infection and some bacterial cultures produce sanguinarin when treated with fungal extracts (McCUE *et al*., 2006).

Vegetal extracts containing alkaloids have been used since ancient times. Socrates, the famous philosopher, for example, was executed by the ingestion of a cicute-based beverage, rich in coniin alkaloids. Amazon indians use the dry extract of a plant known as curare, containing the alkaloid tubocurarin, to poison their arrows for hunt and war. Some cultures have also adopted hallucinogenic alkaloid consumption on their rituals (EL-SEEDI, 2005).

The first scientific works about the bioactive properties of alkaloids were the “opium salt” description by Derosne in 1803 and “principium somniferum” by Serturner, in 1805. Morphin structure was determined later, in 1923 by Robinson and Gulland. Other isolated alkaloids examples are: stricnin (rodenticide), emetin, escopolamine and

papaverin. More recently even antitumoral agents with alkaloid structure were found, as those extracted from *Cantharantus roseus* (HENRIQUES *et al*.; 2003).

First alkaloid extractions were conduced by van Leeuwenhoek, in 1678. Due to its alkaline character, alkaloids have chemical properties similar to those of ammonia. They form soluble salts in acidic solutions and precipitate, as free amines in alkaline solutions, with the exception of quaternary ammonium containing molecules, wich have different properties.

Those chemical properties can be explored for alkaloid extraction. Two methods are the most used: alkaline or acidic medium extraction. In both cases a low polarity solvent pre-treatment must be carried in order to eliminate lypophyllic substances. N-hexane or petroleum-ether can be used (HENRIQUES *et al*.; 2003).

Diethyl ether extraction pre-treatment was used for the present experiment. It was followed by an alkaline medium extraction: a strong basic solution (KOH 5M) and a saturated NaCl solution were added to pre-treated filtered broths in order to precipitate the alkaloids, which were then recovered by extraction with chloroform.

2.2.1.2 Quinones

Quinones are organic compounds with two carbonilic groups and at least two double bonds in their carbon skeleton. They can be considered as phenols oxidation products. Conversely, quinones reduction render the respective phenols. Some of the quinones, as naphto, anthra and phenantraquinones can be classified as aromatic compounds (FALKENBERG, 2003).

Quinones are usually yellow or orange cristaline substances, but occasionally they can be blue, green or black. Their contribution for tissue colours is less significant than that of carotenes and anthocianins, but plants that contain quinones have been traditionally used as a natural source of dyes. There are evidences that quinone dyes were used since ancient times at Egipt, Persia and India. They have perceived that animals fed with alizarine, an anthraquinone obtained from the roots of a plant (*Rubia tinctorum*), induced the development of red coloured bones (IRVAN *et al*., 2006).

Quinones are strong oxidizing agents. Their original role in metabolism is related to electron transferences, mainly associated with both prokariothic and eukariothic cellular respiration. Plastoquinones and philoquinone (K_1 vitamin) are primary metabolites, probably found in all photosynthesising tissues. Ubiquinones are found in most eukariothic organisms.

It is generally accepted that certain quinones have defense roles in plants, protecting them from insects and other pathogens. They also are believed to act as allelopathic substances, i.e. inhibiting competitor vegetals growth at the surroundings of the producer plant (FALKENBERG, 2003).

Quinone containing vegetals are mainly used for their laxative activity, but some of them were also found to have other important biologic activities.

Conocurvone, for example, is a trimeric naphtoquinone that shows inhibitory activity over HIV virus replication. Another example are *Avicennia* naphtoquinones, wich have shown strong protective antineoplasic properties.

Diverse naphtoquinones, present on different species of vegetals, have shown activity against *Leishmania*. Benzoquinones, as primine and perezone, and naphtoquinones, as β-lapachone, mansonones A, C, E and F presented activity against trypanosomatidae. Other quinones, specially o-naphtoquinones have shown in vitro activity against trypomastigote forms of *Trypanosoma cruzi*. Diospirine, wich have shown antiparasitic activity against promastigote forms of *L. donovani*, was chemically modified to reduce its toxic effects.

Quinones are fairly soluble in acetone and chlorophorm, but chlorophorm dissolves polymeric quinones better. Grinding, percolation or both can be applied as extractive procedures. The standard method for quinones extraction involves two steps: a methanol:chlorophorm mixture (2:1 v/v) extraction, followed by a purification with a water/hexane partition (HU, FUJIE & URANO, 1999). Other approaches have been recently proposed, as supercritical CO_2 extraction, with the objective of elliminating or at least reducing hazardous solvents utilization (IRVAN *et al*., 2006).

2.2.1.3 Terpenes

Terpenoids constitute a wide spectrum of substances that share the distinctive feature of being derived from isoprene. Isoprene, by its turn, originates from mevalonic acid. Carbon chains on terpenoids result from the fusion of different numbers of pentacarbonated isoprene units.

There are more than 8000 known terpenic compounds, and more than 1150 of these are found in volatile oils. The most common terpenes in volatile oils (90%) are monoterpenes (terpenes with only one isoprene unit). Sesquiterpenoids are composed of three isoprenoid units; triterpenes, six and tetraterpenes, eight. Terpenes can also be subdivided based on the presence or absence of ciclic structures and functional groups, as alchools, lactones or aldehydes (PADUCH *et al*., 2007).

Although initially not associated with any function, now these substances are known to be related with the attraction of pollinizers, water retention, allelopathy (inhibition of competitor species) and protection against predators in plants. Authors have demonstrated that the toxicity of some of these substances effectively confer protection against predators and infestants.

Main extractive procedures are: enfleurage, steam drift (possibly the most utilized), organic solvent extraction (usually ether or dichloromethane), simple pressing and supercritic CO_2 extraction (SIMOES & SPITZER, 2003).

2.2.2 Antileishmanials

Although there are nearly 25 known antileishmanial compounds, only a few are used for humans, and most of these must be administered parenterally.

Leishmaniases chemotherapy is primarily based on pentavalent antimony containing drugs and secondarily on pentamidine and amphotericin B. But some *Leishmania* spp*.* strains resistence to antimonials have been reported since 1940s as a serious public health problem (WONG & CHOW, 2006).

The oldest antileishmanial was urea stibamine, developed in India in 1922. It had severe toxic effects. However its, less toxic, pentavalent compounds were the sole

treatment modality for several decades, and saved millions of lives. But, since the 1970s unresponsiveness to pentavalent sodium antimony gluconate (SAG), even at high doses, started to be reported. This led to clinical trials of pentamidine and amphotericine B, with relative success. Amphotericine B, an antifungal compound, was also found to be highly nephrotoxic. To minimize these side effects various safer, but exorbitantly costly, colloidal and lipid formulations have been prepared.

More recently, focus has been given to finding oral drugs to minimize injection-associated complications. Many substances, including antifungal ketoconazole, were reported effective (SAENZ, PAZ & BERMAN, 1990).

Lichen galactomannans had shown leishmanicidal activity, *in vitro*. This activity was improved by complexation with vanadium ions. Those polysaccharides have shown immunomodulating activity over macrophages, suggesting action by both mechanisms: direct citotoxicity and stimulation of the immune system (NOLETO *et al*, 2001).

The most promising drug found is notoriously also an anticancer compound, miltefosine (Impavido®), that belongs to the alkylphosphocholine group. It is the first oral antileishmanial drug available (WHO, 2002). It was found to be 94%–97% effective in experimental and clinical trials. Another good aspect is that it does not require refrigeration. India government hopes to erradicate visceral leishmaniasis of their country until 2010, with miltefosine (WHO, 2008d). On the other hand, this drug cannot be given during pregnancy, shows severe gastrointestinal side effects and its cost can be a limiting factor (SINDERMANN *et al*., 2004).

Paromomycin, allopurinol, and sitamaquine, altough not so effective as miltefosine, are also antileishmanial substances and have been reported with variable cure rates. Combination therapies (combined antiparasitic substances) are currently being considered, especially to avoid parasite resistence development against miltefosine. Some drugs that were already tried include atovaquone, roxithromycin, and edelfosine (SINGH & SIVAKUMAR, 2004).

Drug development for leishmaniases seem to be an excessively slow process, possibly due to a lack of financial return expectative, because most affected areas are at developing countries (STROM, 2006). Technology probably represents the only possibility of solution for this challenge. The present experiment is part of this effort.

2.2.2.1 Glucantime® (positive control)

For the present experiment, *N*-methyl meglumine antimonate (Glucantime®), wich is a pentavalent antimonial, was used as a positive control substance against leishmaniasis. This is a substance with assured activity and was used as a reference for evaluation of the tested mushroom extracts.

Glucantime® (FIGURE 9) is a commercially available drug, widely prescripted as the initial treatment for cutaneous and sometimes for visceral leishmaniasis. This stills an aggressive chemotherapeutic substance. At least 34% of the patients treaten with pentavalent antimonials suffer serious side effects (ANTINORI *et al*., 2008). Side effects of this medication include hematologic, renal, hepatic, pancreatic and cardiac toxicity, that can even lead to death (HAJLAOUI *et al*., 2008).

FIGURE 9 - GLUCANTIME FLASKS. SOURCE: Disanejercito (2008).

Meglumine antimonate is usually administered by intramuscular injections, in a concentration of 300mg/mL. Injections are known to cause many side effects in most patients. In 2002, Farmanguinhos-Fiocruz, a pharmaceutical company that belongs to the Brazilian Ministry of Health, developed an alternative process for obtaining meglumine antimonate, due to an unsupplied market (MORAISTEIXEIRA *et al*., 2008).

Although working for many cases, this medication´s serious side effects and reported parasites resistance cases demonstrate the need for new, better antileishmanial drugs development (WHO, 2008d).

2.3 ANTIPARASITICS SCREENING

Many *in vitro* and *in vivo* methods are actually available to assess antiparasitic activity. Each of them has advantages and drawbacks. *In vitro* tests, for example, although not using animals, are limited for previewing complex interactions that naturally happen in living environments. On the other hand, *in vivo* tests, although giving results more approximated to human reactions, can´t be simply extrapolated.

In vitro tests are based on protozoa cultivation and evaluation of their survival or of alterations on their metabolism upon test substances addition.

One simple method for assaying protozoa survival is to observe if their motility is significantly reduced or stoped, with an optical microscope.

Other methods involve the assimilation of radioactive isotopes by protozoa as they grow. Measuring the incorporated radioactive compounds concentration after an incubation period allow to infer protozoa growth rate. Tritiated thimidine is a current methodology of this type.

Alternatively, colorimetric reagents that change their color as they are metabolized can be used to measure metabolism rhythm alterations. Examples of these reagents are MTT, XTT and WST-1, which are tetrazolium salts that change color when metabolized. Color intensities can be measured with a spectrophotometer and are proportional to parasite metabolism rate (WILLIAMS *et al*.; 2003).

2.3.1 MTT

MTT, wich is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is a yellow tetrazolium salt that turns purple when reduced (cleaved) to formazan compounds. It is useful for many bioassays, because cellular metabolism can reduce MTT. Cellular metabolism rate can be inferred by the color of the medium after incubation with this reagent (ROCHE, 2008).

As formazan salts are water-insoluble, they must be properly solubilized before reading (10% SDS, 50% isopropanol can be used). Color intensity measurement is

accomplished with a spectrophotometer. For microassays, a 96-well plate reader is better suited (ROCHE, 2008; ROCHE 2008b).

It is currently used for measuring factor-induced cytotoxicity or cell necrosis, but cell viability can also be assayed. And since proliferating cells are metabolically more active than non-proliferating cells, these tetrazolium salt-based assays are also frequently used to measure cell activation and proliferation (ROCHE, 2008; ROCHE, 2008b). Current applications include:

- Evaluate growth-inhibitory or cytotoxic effects of compounds.
- Analyse cytotoxic and cytostatic effects of potential drugs.
- Analyse cytopathic effects of viruses and screen compounds with potential anti-viral activity.
- Screen antibodies for growth-inhibiting potential.
- Study the effects of substances over immune system cells (proliferation or suppression).

There are other tetrazolium salts also used for similar applications, with small variations (XTT and WST-1). Many bioactive compounds screening studies have already been performed with those reagents.

Those reagents have the advantage of being non-radioactive. Methodologies using them are easy to perform and fast to read. Drawbacks are that sensibility and accuracy are not as high as with DNA based methods (radiolabelled thymidine or bromodeoxyuridine).

MTT was already used for antileishmanials screening. The protocol used for the present experiment was adapted from Estevez *et al*. (2007).

2.3.2 Radiolabelled thymidine

Cell proliferation can be measured with radiolabelled thymidine methodologies. This is based in the fact proliferating cells incorporate free nucleotides, as thymidine, when this is present in the culture medium.

Thymidine molecules, synthesised with radioactive isotopes (^{3}H or ^{14}C), are added to culture medium. These are called radiolabelled thymidine molecules. As cells

multiply, their DNA is replicated. Radiolabelled thymidine molecules are incorporated through this process.

After some incubation time cells are collected with a cell harvester, being transferred to a filter paper and nonincorporated radiolabelled thymidine molecules are washed. Pieces of the filter paper containing cells are transferred to a tube and immersed in a scintillation solution.

Radioactivity emission intensity of each tube content is measured with a scintillator and compared with the radioactivity emited by the total concentration of thymidine initially added.

Many research groups have used radiolabelled thymidine methodologies for assessing substances bioactivities against tumour cells, immune system cells and even parasite cells (ROCHE, 2008).

These methodologies are very elegant and have a really high sensibility. The main drawback is that they involve radioactivity manipulation. An alternative methodology of DNA synthesis quantification involves the utilization of BrdU (bromodeoxyuridine), wich is also a modified, but nonradioactive nucleotide that incorporates to newly synthesised DNA molecules. For BrdU, application of immunostaining techniques allow the method to be colorimetric or by fluorescence measuring (ROCHE, 2008c).

2.4 MACROMYCETES

Mushrooms are also called “Macromycetes”, a latin name that designate fungi that are visible with the naked eye; in opposite to the term “Micromycetes”, wich designate the microscopic fungus. Most of the mushroom forming fungi are basidiomycetes, because their spores are produced over the top of microscopic protuberant structures named basidia. A fewer number of mushrooms, called ascomycetes, produce their spores inside sac structures named asci (PUTZKE & PUTZKE, 2002) (FIGURE 10).

Fungi are achlorophylated eukariotic organisms. They are heterotrophic, absorbing extracellular nutrients, previously degraded by excreted enzymes. They can degrade and utilize almost any source of organic matter, being essentially important from an ecological perspective.

Macromycetes life cycle has been deeply studied and its general principles are well stabilished. The fruiting bodies are constituted mainly by binucleated cells, with haploid nucleus. At special structures, basidia in the basidiomycetes and asci in ascomycetes (FIGURE 10), cariogamy takes place, generating uninucleated cells, with diploid nucleus. Those uninucleated cells generate haploid spores.

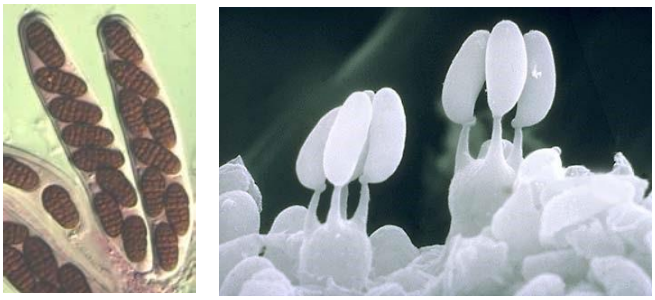


FIGURE 10 - ASCI (LEFT) AND BASIDIA (RIGHT).
SOURCES: Minter (2008); Brown & Brotzman (2008).

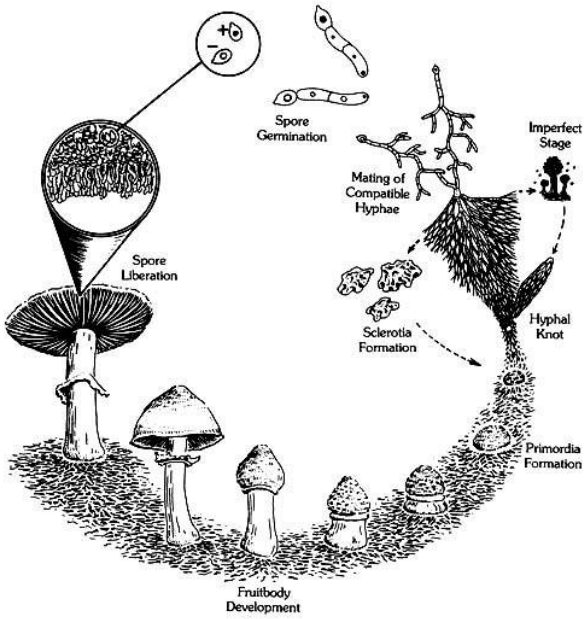


FIGURE 11 - MACROMYCETES LIFE CYCLE. SOURCE: Fungi Perfecti (2008).

Each spore, when environmental and nutritional conditions are favorable, germinate, originating a filamentous structure, formed by uninucleated haploid cells, named primary mycelium (FIGURE 11). When compatible primary mycelia meet, a cellular fusion event happens, originating a binucleated filamentous structure, named secondary mycelium. This secondary mycelium can grow while environmental and

nutritional conditions are favourable. When nutritive resources are finished or environmental conditions get extreme (wet or cold), secondary mycelia organizes to form tissues, which originate fruiting bodies, capable of recombination events and spore dispersion, beginning the cycle again. Spores are relatively resistent and can remain viable for long periods until adequate conditions reemerge.

Reproductive structures (basidia and asci) are located at mushrooms hymenophore, that can be gilled, pored, with teeth or even closed inside spheroid fungi. Spores can leave the fruiting bodies by a series of active and passive mechanisms, as balistic mechanisms, carried by the wind, by water or by animals. Mushrooms have evolved many effective spore dispersion habilities.

Their structure is mainly related both with spore dispersion strategies. holding spores. From hymenophore design, adapted for optimizing spore formation surface, to mushrooms colors, taste and aroma, adapted to atract spore dispersing animals, almost every aspect of macromycetes structure is related with spore dispersion. Fruiting bodies have essentially a reproductive function.

Mushroom identification is classically based on macro and micromorphological analysis, sometimes aided by chemical reagents. Almost every detail of fungal anatomy is full of taxonomic meaning. Some subspecies are very difficult to identify, as do some species and even genera. Classification of these organisms is also a difficult task. This probably happens because unexplored fungal diversity is still very large. It is expected that less than 5% of fungi species are known (PACCOLA-MEIRELES, 2002).

Molecular methods are useful for taxonomiy. Techniques, such as RFLP and RAPD have been applied for identification of industrial strains and monitoring of genetic improvement programs. The most powerful and widespread molecular technique actually used for species identification consists of sequencing short segments of the genomes. These segments must be carefully chosen as they must have some interspecific variability and some intraspecific conservation, i.e. permitting taxonomic associations. Many genomic regions have been studied and now it is believed that rDNA Internal Transcribed Spacers (ITS) are very useful for fungal taxonomy (BINDER & HIBBET, 2003; CAETANO-ANOLLÉS, TRIGIANO & WINDHAM, 2001).

Knowledge and control of fungal processes can literally unlock a whole universe of possibilities out of simple, sometimes otherwise unuseful, biomass, such as agroindustrial residues (LEIFA, SOCCOL, PANDEY, 2007).

Fungi and vegetables produce much more secondary metabolites than animals. This is a characteristic that makes mushrooms a rich source of bioactive substances. Organic acids, polysaccharides, plant growth regulators, alkaloids, mycotoxins, and antibiotics are some examples of bioactive substances already produced by filamentous fungi at industrial scale (EL-ENSHASYA, 2007).

Traditional culture of many countries attribute curative properties to mushrooms. Some of these popularly acclaimed properties have proven true under experimental conditions (SOCCOL *et al*., 2006).

Although Brazil has an admirable fungal diversity, brazilian traditional culture on mushrooms is relativelly scarce in comparison to some european and asian countries, and even Mexico or the United States. Not many local mushroom species have been used for nutritive or medicinal purposes. Most of the species comercialized in Brazil nowadays have been imported from other countries. Exceptions are *Agaricus subrufescens* (= syn. *A. brasiliensis*) and *Pleurotus djamor*. *A. brasiliensis* is used mainly for medicinal purposes and *P. djamor* is a distinctive pink edible species. Although produced in an industrial scale, those species have a very restricted market relatively to *Agaricus bisporus* (Paris mushroom) or *Lentinula edodes* (shiitake).

Mushroom cultivation and processment are well stabilished industrial branches, especially in asiatic and european countries. More than 10 species are cultivated at large scales there. Mushroom products include fresh, dried or condimented fruiting bodies, besides food additives, pharmaceuticals and nutraceuticals (SOCCOL *et al*., 2006).

Macrofungi are now well known as important natural sources of immunomodulating and anticancer agents. Substances as β-D-glucans and triterpenes have repeatedly shown strong bioactivities, experimentally (MORADALI *et al*., 2007). Some of these substances have already passed all test phases and are being produced for commercialization. Examples include polysaccharopeptides (PSP and PSK, named Krestin commercially), extracted from *Coriolus versicolor*; and the polysaccharides Lentinan, extracted from *Lentinula edodes*; Schyzophillan, extracted from *Schyzophillum*

commune; Grifolan, from *Grifola frondosa*; *Agaricus brasiliensis* β-D-glucans; among many others. Some of these substances are being prescripted by phisicians as complements to cancer chemotherapy (ZAIDMAN *et al*., 2005).

Many mushroom proteins have been reported to activate lymphocytes and to stimulate cell proliferation. The activation of immune cells can inhibit the growth of implanted tumor cells or prevent infections, for example. However, some others are cytotoxic or have immunosuppressive effects (SHEU *et al*., 2004). Those can be used directly against cancer cells or parasites.

Some substances, as alkaloids, are believed to protect fungi from infections and parasites (PANACCIONE, 2005). There are evidences of antiparasitic effect on some substances, as polyporic acid (2,5-dihydroxy-3,6diphenyl-1,4-benzoquinone), isolated from *Hapalopilus rutilans*, wich has shown enzymatic inhibition bioactivity (KNECHT, HENSELING and LOFFLER, 2000). Some fungal polysaccharides have also shown some *in vivo* antiparasitic activity, aparently in an indirect form, through the activation of the hosts' immunological response (KOGAN, 2000).

For the present experiment, twelve different macromycete species were cultivated and processed for bioactivity evaluation. Information on their isolation, cultivation and extractive procedures are on the next sections.

Although some of those species are cosmopolites and common, there is a relatively little number of articles on some of them. Articles on the antiparasitic activity of mushroom compounds are almost inexistent.

Even the photographs or popular descriptions of some of the mushrooms used for the present experiment are somewhat difficult to find. This evidences the ineditism of this practical and suggest the vastness of this unexplored field, open for further experimentation. There follows a brief description and review of each macromycete species used for this experiment.

2.4.1 *Auricularia fuscosuccinea* (Montagne) Farlow 1893

Classification: Fungi, Basidiomycota, Agaricomycotina, Agaricomycetes, Auriculariomycetidae, Auriculariales, Auriculariaceae, Auricularia.

FIGURE 12 - *Auricularia fuscosuccinea* FRUITING BODIES IN THEIR NATURAL ENVIRONMENT.
SOURCE: the author (2008).

A. fuscosuccinea (FIGURE 12) is classified among the edible mushrooms of Heterobasidiae, a subclass of Basidiomycetes. The most popular mushrooms of this genus are *A. auricula*, commonly known as 'Tree-ear' and *A. polytricha* or 'Jew's ear'. These mushrooms have been widely used in Chinese cuisine since ancient times, and are known for their pharmaceutical effects in traditional chinese and korean medicine.

Immunomodulating activities, such as antitumor activities, have recently been experimentally exhibited by their nonstarch polysaccharide components, especially glucans. *A. auricula* have very high dietary fiber content which may have potential hypocholesterolemic effect similar to other high-fiber foods. *A. polytricha* was found to lower plasma total cholesterol levels in rats.

A. auricula was effective in lowering both the serum total cholesterol and LDL cholesterol levels, without affecting the concentraton of serum HDL cholesterol. The reduction of serum total cholesterol by the mushroom diets is attributable to the fall in LDL cholesterol (CHEUNG, 1996).

Methanol extracts of *A. mesenterica* exhibit antimutagenic activity. Methanol extract of *A. auricula* inhibited lipid peroxidation and decreased liver damage in benzopyrene-treated mice (YOON *et al*., 2003). Immunomodulatory proteins, called APP were isolated from *A. polytricha* and characterized (SHEU, 2004).

Acidic polysaccharides, which exhibited anticoagulant activity, were isolated from *A. auricula* fruiting bodies, after alkaline aqueous extraction. Some korean people

traditionally consumed this species and attributed antithrombotic properties to it. Antioxidant activity was also experimentally attributed to *A. auricula* polysaccharides. With these properties in mind, bread was prepared with an *A. auricula* polysaccharides based flour. It is possible that this species is going to be an important source for the development of new functional food products (FAN *et al*., 2006).

2.4.2 *Ganoderma applanatum* (Persoon) Patouillard 1889

Classification: Fungi, Basidiomycota, Agaricomycotina, Agaricomycetes, Polyporales, Ganodermataceae, Ganoderma.

FIGURE 13 - *Ganoderma applanatum* PARASITIZING A LIVING TREE. SOURCE: the author (2008).

G. applanatum (FIGURE 13) is a perennial white-rot fungi, unedible for its leathery texture and bitter taste. It´s buffy white hymenophore has a brown surface underneath, that appears when it´s surfacial spore layer is scratched.

This species is popularly known as the artist´s conk or artist´s polypore, because anyone can make drawings on it´s hymenophore, simply scratching it gently with a sharp point (LÆSSOE & LINCOFF, 1998).

It presents a necrotrophic relation to its hosts. That is, this species infects living trees, parasitize them until they die, and persists degrading the host´s tissues, even after death (PACIONI & LINCOFF, 1992).

Ganoderma species, as *G. lucidum* and *G. applanatum*, have been used in traditional Chinese and Japanese medicine for their attributed pharmacological properties as control of blood pressure, treatment of chronic bronchitis, and also as an immune enhancer and stress reducing agent (MING *et al*., 2002).

G. applanatum fruiting body has been used as traditional medicine in China for anti-cancer, anti-virus, and immuno-stimulation physiological activities. Some of those bioactivities, such as anti-tumor and immunomodulation were already scientiffically proven (LEE *et al*., 2007). More than 100 triterpenes were isolated from *Ganoderma* species and their cultured mycelia. Some of them with significative bioactivities, such as cytotoxicity against hepatoma cells *in vitro* (CHAIRUL *et al*., 1991). In the case of *G. applanatum*, the triterpenes ganoderenic acid and applanoxidic acid derivatives have been previously isolated (MING *et al*., 2002).

Liquid-cultured mycelium of *G. applanatum* has been reported to contain useful anti-tumor and immunomodulator polysaccharides, evaluated by *in vitro* and *in vivo* experiments. Polysaccharide production by *G. applanatum* submerged fermentation was already optimized by Lee *et al*. (2007).

G. applanatum is one of the few currently known sources of active βGlucuronidase enzyme. The production of this enzyme by *G. applanatum* submerged cultivation was optimized by Chouiter, Roy and Bucke (2007).

2.4.3 *Ganoderma australe* (Fries) Patouillard 1889

Classification: Fungi, Basidiomycota, Agaricomycotina, Agaricomycetes, Polyporales, Ganodermataceae, Ganoderma.

Ganoderma australe is a perennial bracketlike fungus, known to cause white-rot on wood. It is evolutively very near to *G. applanatum*, sharing many characteristics (RIGAS *et al*., 2007). *Ganoderma* spp. are known for their ligninolytic potential, wich is evidenced by those species avidity for their natural substrate.

Logs infected by *G. australe* mycelium are known as “palo podrido” or “huempe” on south Chile (South America). As the enzymatic process this macromycete operates improves wood digestibility for animals, those decayed logs are then consumed by cattle in the rain forest. This selective delignifying activity is being studied for it´s biotechnological applications (FERRAZ *et al*., 2001; ELISSETCHE *et al*., 2006; TERRÓN *et al*.; 1995). *G. australe* has also been studied for its potential to degrade the

organochlorine inscticide lindane in liquid agitated cultures and in solid substrate composed of a soil and wheat bran mixture (RIGAS *et al*., 2007).

White-rot fungi had been studied for bioremediation purposes due to their powerful enzymatic systems. Some of the enzymes produced by *Ganoderma* spp. are lignin peroxidase, manganese peroxidase, and laccases, which are frequently referred to as lignin-modifying enzymes (LMEs). Rigas *et al*. (2007) detected genes intimately related to lignin peroxidase in *Ganoderma applanatum and G. australe* (FIGURE 14).

FIGURE 14 **-** *Ganoderma australe* GROWING ON DEAD WOOD. SOURCE: the author (2008).

2.4.4 *Ganoderma stipitatum* (Murrill) Murrill 1908

Classification: Fungi, Basidiomycota, Agaricomycotina, Agaricomycetes, Polyporales, Ganodermataceae, Ganoderma.

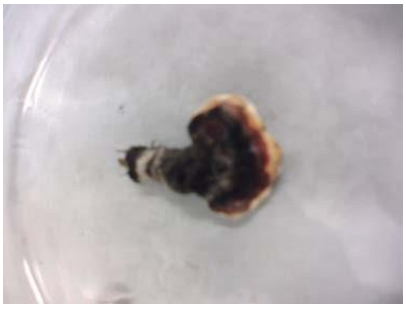


FIGURE 15 - *Ganoderma stipitatum* FRUITING BODY OVER PETRI DISH. SOURCE: the author (2008).

Although some species of this genus are very studied, and have notable medicinal properties, this particular species is almost unknown. No previous records of scientific articles on this species were found.

This species aspect is very similar to *G. lucidum*, but smaller (FIGURE 15). Consistency is also very leathery. These *Ganoderma* species are all unedible because of

context rigidity and bitter taste. By the other hand, many nutraceutical products are being currently produced with *G. lucidum* extracts, powders and infusions.

G. stipitatum is a promisor species, because it is both almost unexplored and taxonomically very approximate to pharmacological substances rich species, as described in sections 2.4.2 and 2.4.3.

Although difficult to isolate, because of its fruiting bodies' small size and hard consistency, its mycelium is very strong and relatively easy to cultivate. In nature, it can appear sole or in small groups of fruiting bodies over wood or soil. It´s natural substrate is lignocellulosic, with necrotrophic or saprotrophic behaviour. This species is probably interesting for medicinal products and enzymes prospection.

2.4.5 *Lepista sordida* (Schumacher) Singer 1951

Classification: Fungi, Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricomycetidae, Agaricales, Tricholomataceae, Lepista.

Lepista is a widespread genus, with many edible species, as *L. nuda*, *L. saeva* and *L. sordida* (FIGURE 16). Those mushroom species are relatively popular in european countries, specially western european countries and France. As some of them have some commercial potential, as edible species, and are somewhat difficult to differentiate by morphological description, some molecular techniques have been developed for their identification (STOTT, DESMERGER & HOLFORD, 2005).

FIGURE 16 - *Lepista sordida* FRUITING BODY OVER ITS NATURAL SUBSTRATE. SOURCE: the author (2008).

Two bioactive diterpenes, called lepistal and lepistol, have been isolated from *L. sordida* mycelium. They exhibited modulatory and cytotoxic activities over human lymphoma and leukaemia cells *in vitro*. Lepistal also have antimicrobial activity against a broad spectrum of bacteria and fungi species (MAZUR *et al*., 1996).

L. nuda is edible and also used in traditional medicine. It contains Vitamin B1. Infusion of this macrofungus is used for preventing beriberi and its decoction is used for the treatment of abscesses and wounds. Polyphenol oxidase enzymes production by this fungus were researched for industrial applications (COLAK *et al*., 2007).

L. nuda and *L. sordida* can grow in fairy rings, circles of fruiting bodies that emerge from radially grown mycelium. Organic chlorine compounds are known to be synthesised by *L. nuda* mycelium during organic matter degradation (HJELM, JOHANSSON & ÖBERG, 1999).

Antioxidant activity was found on methanolic extracts of *L. nuda*. Antioxidant mechanisms were found to be attributed to a strong hydrogendonating ability, a metal-chelating ability, and effectiveness as good scavengers of superoxide and free radicals. Phenolic compounds seem to be the main compenents responsible for this activity (ELMASTAS *et al*., 2007). Those methanolic *L. nuda* extracts also exhibited antimicrobial activity against some bacteria strains (DULGER, ERGUL & GUCIN, 2002).

2.4.6 *Lycoperdon marginatum* Vittadini 1842

Classification: Fungi, Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricomycetidae, Agaricales, Lycoperdaceae, Lycoperdon.

Those field macromycetes grow on organic matter rich soil. They have a spherical morphology (FIGURE 17), and their hymenophore is enclosed. When the spores are mature, they can leave the fruiting bodies by an apperture on the top of the sphere or by disruption of the structure by mechanical forces.

The *Lycoperdaceae* family, once thought to pertain to the Gasteromycetes, was reclassified as pertaining to the Agaricales, with the aid of molecular biology techniques (nu-rDNA). It means these organisms are evolutively close to the gilled macromycetes of the *Agaricus*, *Coprinus* and *Lepiota* genera. On the other hand, some species once

classified as pertaining to the genus *Lycoperdon* were reclassified by molecular methodologies as *Morganella* or *Apioperdon*. Those phylogenetic analyses identified at least 31 species of *Lycoperdon* (LARSSON & JEPPSON, 2008).

FIGURE 17 - *Lycoperdon marginatum* ALMOST SPHERICAL FRUITING BODIES. SOURCE: the author (2008).

This family comprises enclosed fruitbodies, filled with a white gleba that turns brown with maturity. They are known as puffballs and some of them are edible when young, when gleba stills white (LÆSSOE & LINCOFF, 1998).

2.4.7 *Oudemansiella canarii* (Junghuhn) Höhnel 1909

Classification: Fungi, Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricomycetidae, Agaricales, Marasmiaceae, Oudemansiella.

FIGURE 18 - *Oudemansiella canarii* fruiting body, SHOWING ITS GILLED HYMENOPHORE (LEFT), AND AT TWO MATURITY STAGES (RIGHT). SOURCE: the author (2008).

O. canarii (FIGURE 18) is an edible and cultivable mushroom. EMBRAPA, a brazilian federal technology institution has already researched the cultivation of this

species over local gramineous substrates, as an adaptation of Jun-Cao technology, firstly developed in China (URBEN *et al*., 2004).

O. platyphylla was found to be a source of lectins (MATSUMOTO *et al*., 2001). *O. mucida* produces an antifungal compound, called mucidin that have shown activity against *Aspergillus niger* and *Saccharomyces cerevisae* in aerobic phase, suggesting that cytochromes a and b are the primary action site of mucidin (SUBIK *et al*., 1974).

O. mucida was also studied for glicolitic enzymes production, useful for biotransformation applications (VOLC *et al*.; 1995).

2.4.8 *Perenniporia martiusii* (Berkeley) Ryvarden 1972

Classification: Fungi, Basidiomycota, Agaricomycotina, Agaricomycetes, Polyporales, Polyporaceae, Perenniporia.

Perenniporia Murrill 1942 is a large, cosmopolitan genus, which has been broadly expanded in the last 20 years as a consequence of the multiple additions of new species or transfer of existing taxa. It also has some described neotropical species (DECOCK & RYVARDEN, 2002).

FIGURE 19 **-** *Perenniporia martiusii* BLACK AND WHITE FRUITING BODIES. SOURCE: the author (2008).

Perenniporia spp. are highly dispersed through the world. Specimens of many different species were found in Uruguay, Cuba, Puerto Rico, Brazil and even in Korea (DECOCK & FIGUEROA, 2000; CHANG & CHOU, 1999).

Those bracketlike mushrooms have a dark and crusty surface (FIGURE 19), with a white and rubbery marging and a very rigid texture. The hardness of their texture make

them unedible and very difficult to isolate. Despite this, *P. martiusii* mycelium is very easy to cultivate and have a really fast growth rate.

P. fraxinophila is an agent of wood decay that affect living trees. Although usually not killing host plants, *P. fraxinophila* infections reduce their growth rate and make them fragile and susceptible to wind and ice (LESICA, ATTHOWE & DUGAN, 2003). This same species mycelium has been cultivated and studied for the production of metalloproteases, useful for therapeutic application against cardiovascular diseases (KIM *et al*.; 2008).

Perenniporia medula-panis was studied for iron-reducing compounds production, useful for degradation of lignocellulosic materials, as the paper bleaching effluents (ARANTES & MILAGRES, 2006).

Six terpenoids, known to be perenniporiol derivatives, were extracted with benzene from *Perenniporia ochroleuca* cultured mycelium, and characterized (INO, HIROTANI & FURUYA, 1984; HIROTANI *et al*., 1984).

2.4.9 *Pleurotus calvescens* (Berkeley) Singer 1957

Classification: Fungi, Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricomycetidae, Agaricales, Pleurotaceae, Pleurotus.

FIGURE 20 - *Pleurotus calvescens* GREGARIOUS FRUITING BODIES. SOURCE: the author (2008).

Similarly to *G. stipitatum*, this species is almost scientifically unexplored. Although pertaining to one of the most studied genera (*Pleurotus*), no scientific papers were found on this mushroom. Many *Pleurotus* species, such as *P. ostreatus*, *P. sajor-caju*, *P. eryngii*, *P. citrinopileatus* and *P. ostreatoroseus* are edible and commercially cultivated. All

Pleurotus species are adapted to lignocellulosic substrates, causing white rot on wood. Common cultivation substrates include sawdust, wheat bran, bagasses, straw, husks and grassy plants.

Most of *Pleurotus* species are edible, but specimens found in nature should be avoided, because can accumulate environment´s toxic compounds.

Even without being comproved as edible, this is a potential nutraceutical source species. Its morphology, color (FIGURE 20) and smell have a commercial appeal. Future researches on its edibility and cultivation should be conduced.

2.4.10 *Pleurotus djamor* (Rumphius ex Fries) Boedijn 1959

Classification: Fungi, Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricomycetidae, Agaricales, Pleurotaceae, Pleurotus.

FIGURE 21 - *Pleurotus djamor* CULTIVATED OVER LIGNOCELLULOSIC RESIDUES. SOURCE: the author (2008).

Pleurotus djamor is a pantropical species of oyster mushroom (JAMES, LIOU & VILGALYS). It is edible and commercially cultivated. It is a whiterot fungi, with a wood decaying nutrition. *P. djamor* is popularly called red (or pink) (FIGURE 21) oyster mushroom. It was introduced in South China from India in the 1990s. Native brazilian species have recently started to be cultivated. Aesthetic appearance and attractive organoleptic characteristics are making this mushroom an edible species with increasing market. Its fruiting bodies are easily cultivable and adapt well to a wide range of lignocellulosic substrates, showing high yields. Exaples of adequate substrates include coffee pulp and wheat straw (SALMONES, MATA & WALISZEWSKI, 2005).

Water, soluble sugars and protein contents of this mushroom are relatively reduced in comparison to other species. In the other hand, polysaccharide content is relatively high (GUO, LIN & LIN, 2007).

2.4.11 *Plectania* Fuckel 1870

Classification: Fungi, Ascomycota, Pezizomycotina, Pezizomycetes, Pezizomycetidae, Pezizales, Sarcosomataceae.

FIGURE 22 - UNCOMMON *Plectania* sp. FRUITING BODY IN ITS NATURAL ENVIRONMENT (LEFT) AND COLLECTED (RIGHT). EASY TO CONFUSE WITH DRY LEAVES. SOURCE: the author (2008).

This fungus was the only ascomycete tested for the present experiment. This confirms the statistics that point there are relatively fewer ascomycetes than basidiomycetes mushrooms in nature (PUTZKE & PUTZKE, 2002).

It´s fruiting body is very distinct both in shape and texture (FIGURE 22). It grows on soil, over decomposed litter and has a ballistic mechanism of spore discharge.

Slight movements, when the asci are mature, launch a chain reaction of spore ejection, suddenly producing a cloud of spores that gradually disperse.

Some carotenoids, named plectaniaxanthins, produced by *P. coccinea* were purified and characterized, for taxonomic purposes (RØONNEBERG *et al*., 1982;

VACHERON *et al*., 1970; ARPIN & JENSEN, 1967). Those carotenoids were also found on the yeast *Cryptococcus laurentii* (BAE *et al*., 1971), suggesting evolutive proximity.

2.4.12 *Pycnoporus sanguineus* (Linnaeus) Murrill 1904

Classification: Fungi, Basidiomycota, Agaricomycotina, Agaricomycetes, Polyporales, Polyporaceae, Pycnoporus.

FIGURE 23 - ORANGE *Pycnoporus sanguineus* FRUITING BODIES, GROWING ON DEAD WOOD. SOURCE: the author (2008).

This is a white rot fungus (WRF), with pronounced lignocellulosic materials degradation capabilities, attacking certain types of wood (FIGURE 23) in the forests of tropical and subtropical areas of the northern and southern hemispheres (SMÂNIA, 1995). The production of some laccases by submerged mycelial culture of this species was found to be specially interesting for bioremediation applications, such as decolorisation and detoxification of some dyeing products (TROVASLET *et al*., 2007).

P. sanguineus laccases were proven efficient in degrading indigo blue dye, extensively used in clothes industry (BALAN & MONTEIRO, 2001).

As those laccases had shown desirable enzymatic characteristics, as thermostability (LITTHAUER *et al*.; 2007) and optimal activity temperature range between 20 and 60ºC, they have attracted interest for industrial applications. *P. sanguineus* laccases were already produced in a molasses based medium and were even immobilized on magnetic chitosan microspheres for improving their reutilization and stability (JIANG *et al*., 2005).

Invertases are other interesting class of enzymes that are produced by *P. sanguineus*. Those enzymes attack a wide diversity of substrates, but preferrably those

containing fructose, including sucrose, raffinose, stachyose, inulin and levan. Invertases were purified from *P. sanguineus* liquid cultivated mycelium and characterized by Quiroga, Vattuone and Sampietro (1995).

When cultivated in mediums containing glucose polymers as the only carbon source, *P. sanguineus* mycelium produces α-amylases enzymes. When cultivated in the proper mediums, this fungus can excrete a wide variety of economically interesting enzymes, such as xylanases, glucosidases and cellobiohydrolases (SIQUEIRA, MIZUTA & GIGLIO, 1997). *P. sanguineus* was found to be an effective biosorbent for the removal of Pb^{2+}, Cu^{2+} and Cd^{2+} heavy metals from aqueous solutions when applied in fixed bed columns (ZULFADHLY, MASHITAH & BHATIA; 2001).

This fungus has been used since ancient times in popular medicine by indigenous tribes of the Americas and Africa for treatment of some diseases. It was also used for treating skin lesions by certain rural communities of Santa Catarina state, at south of Brazil (SMÂNIA *et al*.; 1995).

Acetone and n-hexane extracts of *Pycnoporus sanguineus* lyophillized mycelium had shown antibacterial activity against *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus* and other species of *Staphylococcus* (SMÂNIA *et al*.; 1995).

3 OBJECTIVE

The aim of this experiment was to search for antiparasitic substances, with direct activity against three different *Leishmania* species, on the mycelial liquid culture broth of twelve distinct macromycete species.

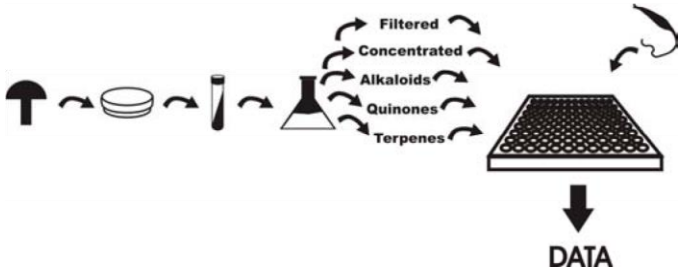


FIGURE 24 - SIMPLIFIED EXPERIMENTAL FLOWSHEET: MACROMYCETE ISOLATION, CONSERVATION, SUBMERGED CULTIVATION, EXTRACTIVE PROCEDURES AND BIOASSAYS.
SOURCE: the author (2008).

Filtrated fermented liquid broth, a concentrated form and three diferent extracts of each mushroom species were tested. Extractive procedures took into acount some of the most promising chemical classes of natural molecules with antiparasitic activity known: alkaloids, quinones and terpenes (FIGURE 24).

4 MATERIAL AND METHODS

4.1 MACROMYCETE STRAINS

The twelve macromycete species used for this experiment were isolated from wild fruiting bodies (table 1). Eight of them were collected from Curitiba´s urban environment, including streets, parks and the Centro Politécnico campus. The other four were collected from the Atlantic Forest in Guaraqueçaba, a brazilian city that pertains to Paraná state and is located at the atlantic seacoast in the south region of the country. Collections were performed by Francisco Vítola, Marcelo Fernandes and Dr. Carlos Soccol.

TABLE 1- MACROMYCETES THAT WERE TESTED FOR THEIR ANTIPARASITIC ACTIVITIES.

SPECIES	ORDER	FAMILY	ORIGIN	SUBSTRATE
Auricularia fuscosuccinea (Montagne) Farlow 1893	auriculariale	Auriculariaceae	Curitiba	Living tree
Ganoderma applanatum (Persoon) Patouillard 1889	polyporale	Ganodermataceae	Curitiba	Decomposed wood
Ganoderma australe (Fries) Patouillard 1889	polyporale	Ganodermataceae	Curitiba	Decomposed wood
Ganoderma stipitatum (Murrill) Murrill 1908	polyporale	Ganodermataceae	Atlantic forest	Decomposed wood
Lepista sordida (Schumacher) Singer 1951	agaricale	Tricholomataceae	Atlantic forest	Soil
Lycoperdon marginatum Vittadini 1842	agaricale	Lycoperdaceae	Curitiba	Soil
Oudemansiella canarii (Junghuhn) Höhnel 1909	agaricale	Marasmiaceae	Curitiba	Living tree
Perenniporia martiusii (Berkeley) Ryvarden 1972	polyporale	Polyporaceae	Atlantic forest	Decomposed wood
Pleurotus calvescens (Berkeley) Singer 1957	agaricale	Pleurotaceae	Curitiba	Decomposed wood
Pleurotus djamor (Rumphius ex Fries) Boedijn 1959	agaricale	Pleurotaceae	Atlantic forest	Decomposed banana tree
Plectania Fuckel 1870	pezizale	Sarcosomataceae	Curitiba	Soil
Pycnoporus sanguineus (Linnaeus) Murrill 1904	polyporale	Polyporaceae	Curitiba	Decomposed wood

SOURCE: The author (2008).

All of these macromycete species were identified by André de Meijer, an expert mycologist and researcher on natural sciences who gently collaborated with this work. This represents just a little sample of the enormous brazilian fungal biodiversity (DE MEIJER, 2006) that still unexplored, and wich constitutes a potential source of useful bioactive compounds.

Identifications were done based on macro and microscopical morphological characteristics, sometimes aided by chemical reactions. All of these isolated species should, for further experiments, be assessed through molecular biology techniques to validate the isolation procedures.

Isolations were performed by Francisco Vítola and Marcelo Fernandes, as described on section 4.2. The isolates used for this experiment were chosen from a collection of more than 40, based on the aspect of the mycelium, adaptation to the formulated liquid medium and to cover the widest range of diversity possible.

4.2 MACROMYCETE STRAINS ISOLATION AND STORAGE

As they arrived at the laboratory, macromycetes fruiting bodies were initially roughly cleaned. Their surface was asperged with alcohol. Then, under asseptic conditions, inside a laminar flow hood, the fruiting bodies were sectioned, and little pieces (1~2mm^3) of their uncontaminated internal tissues were tranferred to Petri dishes containing PDA culture medium.

These Petri dishes containing macromycete tissue pieces over PDA medium were incubated at 25ºC. When the procedure is successful, new mycelia starts to grow from the tissue pieces and take over the whole medium. Growth latency (the time between the isolation procedure and mycelium growth start) varied from less than 24 hours to even two weeks. Sometimes, mycelium didn´t grow at all, because of it´s non-viability or for it´s fastidiousness. Some of the dishes were also unavoidably lost due to fast growing contaminating microrganisms. Eventually, point contaminations occurred, requiring new isolation steps, involving the transference of uncontaminated micelium pieces to new petri dishes. This isolation march continued until pure cultures were obtained. When this was

achieved, mycelium pieces were transferred to tubes (in triplicate) for storage. Only about 40% of the isolation trials were successful.

Tamponed tubes with inclined PDA were the recipient of choice for storing mycelial pure cultures, for it´s little volume and high protection against contamination. From time to time, the cultures need to be transferred to new tubes, as the media get exausted. Storing cultures at 6ºC slow down mycelial metabolism, allowing longer intervals between transferences. With refrigeration, intervals vary between 2 and 6 months, depending on the strain.

Other storage techniques were tested in order to prolong intervals between transferences or even eliminate the necessity of this procedure, but without success. Techniques such as storing mycelium in sterile water, submersion in mineral oil or crioprotector-assisted freezing, all presented reactivation difficulties for most of the species. A protocol that involves freezing of spawn is now being considered.

4.3 SUBMERGED MYCELIAL CULTURE

Mycelia of all the choosen macromycetes were cultured in 250mL of liquid medium, inside 500mL Erlen-Meyer flasks (FIGURE 25), in duplicates. The incubation was carried in an orbital shaker, with agitation and temperature control (25°C, 120 rpm) at least until stationary growth phase (about 30 days). Culture time was decided for each species, based on visual inspection.

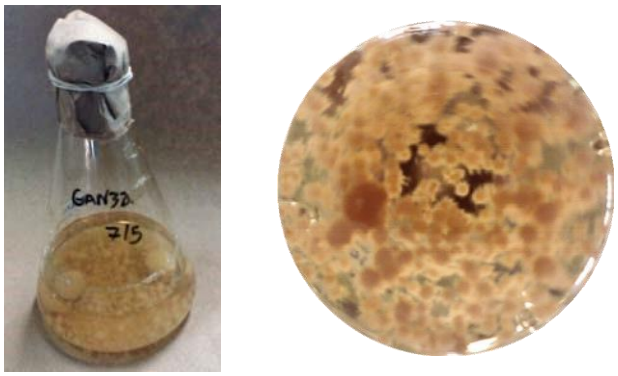


FIGURE 25 - SUBMERGED MYCELIAL CULTURES. SOURCE: the author (2008).

This long incubation period was chosen based on the hypothesis that secondary metabolites accumulation in the liquid broth occur only after the exponential growth phase

of biomass (that usually takes about 15 days for many basidiomycete species, but that can even take 30 days or more for some species). Of course this is a rough approach, but can work for an initial activity assessment of many fungi important bioactive metabolites. Medium composition wasn´t optimized for this experiment. One standard formulation, based on various scientific works and personal experience was adopted. It´s composition is described in TABLE 2.

TABLE 2 - LIQUID MEDIUM FOR MACROMYCETES SECONDARY MYCELIUM CULTURE.

COMPONENT	CONCENTRATION (G/L)
Glucosis	20,00
Yeast Extract	3,00
Malt extract	1,00
$MgSO_4$	0,06
K_2HPO_4	0,06
$CaCl_2$	0,01
pH adjusted to 6,1 with HCl and NaOH addition	

SOURCE: The author (2008).

As bioactivity was detected for some of the candidate compounds, the next step should be the identification of the active principles involved. As these are determined, bioprocess parameters, including process duration, aeration and agitation rates, temperature, pH and medium composition can then be optimized for maximizing active biomolecules production.

4.4 DOWNSTREAM PROCESSES

The broths obtained by submerged cultivation are pools of enormous numbers of different molecules. So there are virtually infinite methods of processing to achieve any of the purified substances and their possible combinations in all the possible concentrations and ratios.

The most important antiparasitic molecules found on natural sources until now belong mainly to three different chemical classes: alkaloids, quinones and terpenes

(KAYSER, KIDERLEN & CROFT, 2002). Extractive steps for this experiment were designed with this in mind, as these classes comprise the most strong candidate molecules for antiparasitic activity tests.

An extractive flowsheet was planned to render the following samples from each of the cultivated macromycetes:

- **filtered** broth;
- **concentrated** filtered broth;
- ether extract (**terpenoids**);
- acetone extract (**quinones**) and
- chloroform extract, with alkalinization of the aqueous phase (**alkaloids**).

The mycelium cultivation resulting liquid broths were filtered with a vacuum filtration system (Buchner funnel and Kitasato coupled with a vacuum pump). An aliquot (30 mL) of each filtered broth was stored as a sample for subsequent bioassays. The remaining filtered volume was divided:

Half of it was extracted with diethyl ether for recovering of terpenoids. The other half was concentrated to about 70mL. 30mL of this concentrated was stored for subsequent bioassays. The remaining volume of concentrated was extracted with 4 volumes of acetone for recovering quinones.

The fraction extracted with diethyl ether consists mainly of organic oils. Although containing all sorts of lipophyllic compounds, these ether extracts are probably rich in terpenes. After ether extraction, the remaining aqueous phase was basified with the addition of 1/2 volume of KOH 5M and salted with 1 volume of saturated NaCl solution for precipitation of alkaloids. Precipitated alkaloids were extracted with chloroform.

Acetone extracts were left for precipitation overnight on the freezer (2ºC) and centrifuged at 5000 rpm for 15 minutes. Precipitates were discarded.

The supernatants are possibly rich in quinones, as these are soluble in acetone.

Evaporation of all solvents was carried at room pressure, under 60°C (with a plate heater) inside an exaustion chamber. All organic solvent extracts samples were ressuspended in PBS after solvent evaporation. The pH of all samples was adjusted to 6,5~7,5 with the addition of HCl or NaOH. After pH adjustment, all samples were filtered with absolute filters (Millipore® 0,22µm), under asseptical conditions, for sterilization.

Therefore, as described, five different processes were applied to each cultivation broth, resulting in five different preparations for each of the twelve cultivated macromycete species. This rendered 60 distinct macromycete derived biocompound preparations.

4.4.1 Filtration

FIGURE 26 - VACUUM FILTRATION SYSTEM. SOURCE: the author (2008).

The mycelium cultivation resulting liquid broths were filtered with a vacuum filtration system (Buchner funnel and Kitasato coupled with a vacuum pump) (FIGURE 26). An aliquot (30 mL) of each filtered broth was stored as a sample for subsequent bioassays. Biomass was discarded. The filtered volumes were in the range between 450mL and 500mL. Supernatant color and smell were characteristic for each cultivated species. Biomass concentration and water retention capabilities also varied markedly, so as the resistance against filtration.

4.4.2 Concentration

After filtration, an aliquot, of approximately 220mL of each filtered broth was concentrated to nearly 70mL by rotoevaporation, i.e. low pressure evaporation, at 75°C. Each sample took about 40 minutes. 30mL from each of the concentrated samples were stored for subsequent experimentation. The remaining concentrated (~40mL) was used for quinones extraction.

4.4.3 Quinones Extraction

Samples (~40mL), previously filtered and concentrated, as described in the previous sections, were added with 4 volumes of acetone (~160mL). Acetone extracts were left overnight (24 hours) for precipitation on the freezer (-2ºC) and, after defreezing, were centrifuged at 5.000 rpm for 15 minutes.

The precipitate was discarded. The supernatant should contain quinones, if they were present in the fermented broths, because those metabolites are soluble in acetone.

4.4.4 Terpenes Extraction

About 220mL of each filtered broth was extracted with 50mL diethyl ether, for recovering the terpenoids. The solvent was added to each sample inside an exhaustion chamber. The flasks containing the aqueous/organic partitions were alternatedly agitated and opened, to release the pressure. After a while, the aqueous and organic phases of all the samples were separated with an extraction funnel (FIGURE 27), also inside an exhaustion chamber.

The fractions extracted with diethyl ether probably consist mainly of organic oils. Although this can contain all sorts of lipophyllic compounds, ether should extract terpenes if they were present in the fermented liquids. The interface was discarded, sometimes within some precipitated material. The aqueous phase was kept for alkaloids extraction, described in the next section.

FIGURE 27 - EXTRACTION FUNNEL, INSIDE EXHAUSTION CHAMBER. SOURCE: the author (2008).

4.4.5 Alkaloids Extraction

For alkaloids extraction, about 200mL of the remaining aqueous phase of the ether extraction procedure (section 4.4.4) was used. One volume of NaCl saturated solution and ½ volume of KOH 5M was added to this aqueous solution. Alkaloids were precipitated in this saline/alkaline condition, and after recovered with 50mL of chloroform, an apolar solvent. As for the ether extraction procedure, chloroform was added to the samples inside an exhaustion chamber. The flasks containing the aqueous/organic partitions were alternatedly agitated and opened, to release the pressure. After a while, the aqueous and organic phases of all the samples were separated with an extraction funnel (FIGURE 31), also inside an exhaustion chamber. Differently from ether, chloroform is heavier than water, so it leaves the extraction funnel before the aqueous phase. Interface and water phase were properly dilluted and discarded.

4.4.6 Solvents Evaporation

Evaporation of all solvents was carried at room pressure, under 60°C (with a plate heater) inside a exaustion chamber (FIGURE 28). At first, solvents were evaporated inside 600mL flasks and finally transferred to 40mL flasks. Ether and chloroform extracts were evaporated to almost dryness, remaining only an oily residue for some samples. Acetone extracts, differently, were evaporated until there was no more smell of organic solvent in the samples (between 1 and 5mL).

FIGURE 28 - HOT PLATE USED FOR SOLVENTS EVAPORATION. SOURCE: the author (2008).

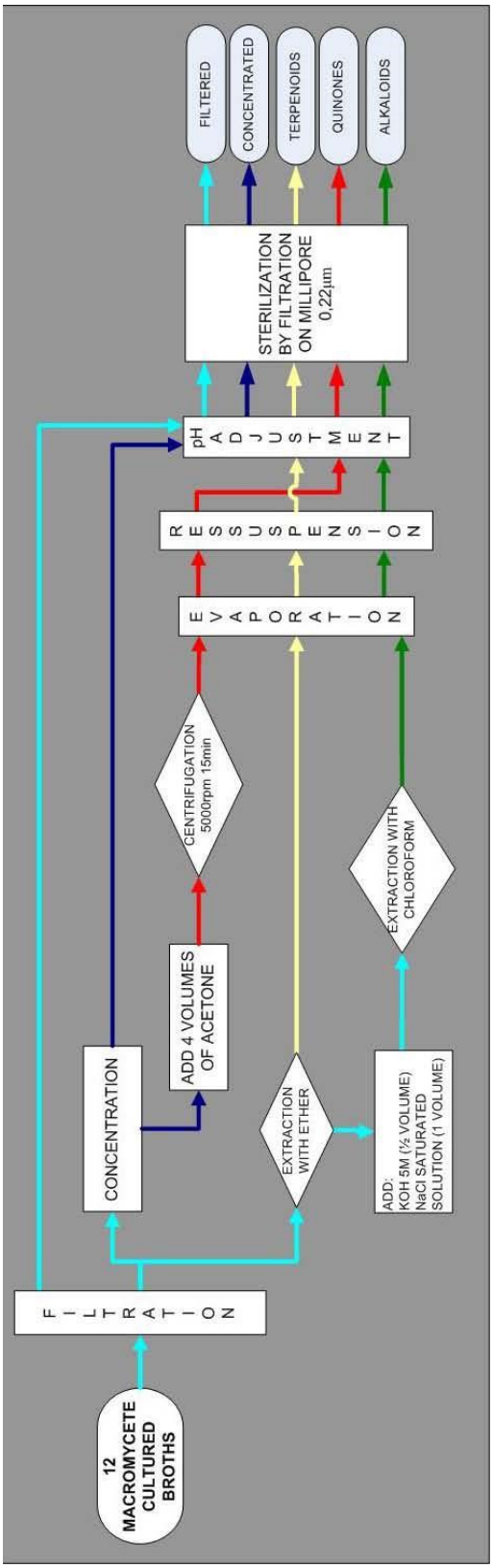


FIGURE 29 – SCHEMATIC FLOWSHEET DESCRIBING DOWNSTREAM PROCESSES FOR THE OBTENTION OF 60 DIFFERENT EXTRACTS FROM 12 DISTINCT MACROMYCETES' SPECIES. SOURCE: the author (2008).

4.4.7 Extracts Ressuspension, pH Adjustment and Sterilization by Filtration

After organic solvent evaporation, samples were ressuspended in PBS, until approximately 30mL. The pH of all samples was adjusted to 7.0≤0.5 with HCl and NaOH. After pH adjustment, samples were filtered through millipore 0,22µm, for sterilization. Filtration was aided by vacuum and conduced inside a laminar flow hood (FIGURE 30).

FIGURE 30 - MILLIPORE FILTRATION SYSTEM INSIDE A FLOW HOOD. SOURCE: the author (2008).

At the end of the process, 60 distinct mushroom extracts samples were achieved. About 30mL of each sample were acconditioned in sterilized glass flasks and stored in a freezer, under -2°C. For the bioactivity assays, the extracts were defreezed, and 2mL aliquots were transfered to sterilized Eppendorf tubes, under asseptic conditions. Downstream processes are summarized in FIGURE 29.

4.5 ANTIPARASITIC EFFECT ASSAYS

Each of the 60 preparations described above was tested against the three different *Leishmania* species: *L. braziliensis*, *L. enriettii* and *L. infantum*.

4.5.1 *Leishmania* spp. strains

Leishmania strains used for this experiment were gently supplied by Dr. Vanete Thomaz Soccol. These are strains standardized for the World Health Organization (WHO) by Dr. Soccol herself. These species were chosen for this experiment because they

represent three different clinical manifestations of the disease: cutaneous (*L. braziliensis*), diffuse cutaneous (*L. enriettii*) and visceral (*L. infantum*). Their identification codes are:

L. (Viannia) braziliensis – MHOM/BR/75/M2903

L. (Leishmania) enriettii (Muniz & Medina, 1948) – MCAV/BR/48/LEM

L. (Leishmania) infantum (Nicole, 1908) – MHOM/BR/74/PP75

4.5.2 *Leishmania* spp. culturing

All leishmania strains were originally stored in liquid nitrogen, under negative 196ºC (-196°C). They were reactivated from liquid nitrogen in NNN (Novy-McNeal-Nicolle) medium (TABLE 4), at 25°C. The three different species of the genus *Leishmania* (*L. braziliensis*, *L. infantum and L. enriettii*) were cultured in three different mediums (CCS, RPMI and modified LIT). *L. enriettii* adapted well to the modified LIT (liver infusion tryptose) medium (TABLE 3), while *L. infantum* adapted better to RPMI. *L. braziliensis* was cultivated on CCS (brain-heart-blood) medium (TABLE 5) because it needed a blood cell supplemented medium. Mediums compositions are described in the following tables:

TABLE 3 - MODIFIED LIVER INFUSION TRYPTOSE (LIT) MEDIUM COMPOSITION.

COMPONENT	CONCENTRATION (G/L)
NaCl	5,0
Na2HPO4	7,5
Tryptose	5,0
Yeast extract	3,0
Brain Heart Infusion	3,0
KCl	0,4
Adjust pH to 7,4 with Na_2HPO_4 1M	
Autoclave at 120°C for 30 minutes.	
Add 2mL hemine (20mg/L) for each L of LIT medium. (previously filtered through 0,2µM millipore filters)	
Add 5mL gentamicin(10mg/mL) for each L of LIT.	
Add 10% FCS, previously decomplemented.	

SOURCE: V. T. Soccol (2008).

TABLE 4 - NOVY-MCNEAL-NICOLLE (NNN) MEDIUM COMPOSITION.

COMPONENT	CONCENTRATION (G/500ML)
NaCl	3,0
bacto agar or pastagar	5,0
Boil for 2~3 minutes.	
Distribute in tubes (4mL/tube).	
Autoclave at 120°C for 30 minutes.	
Add 0,3mL of citrated rabbit blood per tube.	
Let the gelatin solidify in an inclined position inside the tubes.	

SOURCE: V. T. Soccol (2008).

TABLE 5 - CCS (BRAIN-HEART-BLOOD) MEDIUM COMPOSITION.

COMPONENT	CONCENTRATION (G/L)
Brain Heart Infusion	37,0
Bacto agar	15,0
Boil for 2~3 minutes.	
Distribute in 125mL erlen-meyers (10mL/erlen-meyer).	
Autoclave at 120°C for 30 minutes.	
Add 1mL of citrated rabbit blood per erlen-meyer.	
Let the gelatin solidify inside the flasks.	
Add 4mL physiological serum	

SOURCE: V. T. Soccol (2008).

Commercial RPMI (Sigma®) medium, added with 10% FCS was used. RPMI and LIT cultures were conduced inside Roux bottles (FIGURE 31). CCS cultures, inside 250mL erlen-meyers and NNN in tubes. All described species/mediums combinations were maintained inside an incubator, at 25°C.

An inverted microscope was used for monitoring Roux bottles cultures, for their concentration, motility and contaminations. For erlen-meyer cultures, samples were taken for observation on a common optical microscope. Whenever necessary, samples were also took from Roux bottles for better observation on a common optical microscope. Contaminated and old cultures were adequately sterilized and discarded.

Transferrence times were chosen based on parasite countings. Only cultures within a concentration range between 10^7 and 10^9 parasites per mL were repicated. Innoculum rate was of about 20%.

FIGURE 31 - ROUX BOTTLES AND MICROPLATES INSIDE INCUBATOR. SOURCE: the author (2008).

As described by GOMES *et al*.(2008), "…the different *Leishmania* species are not equally easy to cultivate. Contamination is a constant problem, and variations in efficacy among different growth medium formulations or even batches may be encountered". For some assays, NNN medium was found to give better results (CHOUIHI *et al*., 2008).

For this experiment, CCS medium contained rabbit blood cells, wich interfered with the MTT assay. *L. infantum* adapted properly to RPMI medium, as well as *L. enriettii* adapted to the modified LIT medium. In the other hand, *L. braziliensis* didn´t grow well on blood cell free mediums (RPMI and modified LIT). For assaying this species with MTT, a blood cell disruption procedure and medium transference were conduced before applying the bioactivity evaluation methodology.

4.5.3 Blood cell disruption

- 20mL of the liquid fraction of a CCS medium previously cultured with *Leishmania* (10^7 cells/mL) was filtered through a sterilized cotton cloth and transferred to a sterilized Falcon tube under asseptic conditions. The CCS medium solid matrix and the erlen-meyer originally containing it were washed with 10mL of LIT modified medium, wich was filtered and added to the remaining suspension on the Falcon tube.
- 10mL of physiological serum were added and the resulting suspension was centrifuged for 15 minutes at 5000rpm, 25ºC.
- Supernatant was pipeted and discarded.

- 30mL of physiological serum were added to the remaining pellet.
- The resulting suspension was centrifuged for 15 minutes at 5000rpm, 25°C.
- Supernatant was pipeted and discarded.
- The remaining pellet was ressuspended in 25mL of modified LIT medium (10% fetal calf serum).

4.5.4 *Leishmania* spp. counting in Thoma chamber

This technique was used to quantify parasites concentration on cultures liquid. The Thoma chamber consists of a microscopy slide with a thin well of a very precise volume and an imprinted microscopic grid. With the aid of an optical microscope, microrganisms contained in a liquid sample can be counted. The method consists of covering the well with a small glass slide and transferring about 10µL of the properly stained protozoary cultured liquid to be quantified inside the slide well by capillarity. With an optical microscope, individual parasites inside the grid squares (quadrants) are counted. As the chamber volume is known (the volume over each quadricle is of 0,004mm^3), parasites concentration on the sample can be determined.

Leishmania culture samples were adequately dilluted before counting (1/2, 1/5 or 1/10). A fixation/staining solution was used for the dillution, besides it´s main functions (fixation and staining of the parasites).

An equipment consisting of a microscope coupled with a video camera, linked to a monitor was used to aid counting. A manual counter was also used. Microrganisms within 5 quadrants were counted and the mean number was divided by the volume over one quadrant. The values were corrected adequately for previously applied dillutions.

4.5.5 MTT method

L. enriettii was precultured on modified LIT modified medium (10%FCS), *L. infantum* was precultured on RPMI (10%FCS) and *L. braziliensis* was precultured on CCS (10%FCS) medium. Samples were taken periodically to monitorize parasite counts.

Parasite countings were performed with a Thoma chamber, as described in section 4.5.4. When *Leishmania* counts were on the 10^6~10^7 (parasites/mL) range, cultures were dilluted with the respective medium until the 10^5~10^6 (parasites/mL) range. *L. braziliensis* culture was processed, as described in section 4.5.3, in order to eliminate red blood cells and transfer leishmanias to LIT modified medium before dillution.

The applied MTT protocol was adapted from Estevez *et al*., 2007.

Properly dilluted *Leishmania* cultures were distributed in 96-well microplates, 100µL per well. Then, 100µL from each of the 60 macromycete extracts were added to each well (in triplicate) as designed on FIGURE 32. Positive, negative controls and a blank were added in quadruplicate.

Positive control consisted of 100µL Glucantime® (section 2.2.2.1) instead of macromycete extract. Negative control consisted of 100µL culture medium instead of extracts. The blank consisted of 200µL of culture medium only, without *Leishmanias* and without the addition of the extracts.

	PS	G3	AU	PD	PC	LM	PM	PL	GA	LS	OC	GS
Filt.												
Filt.												
Filt.												
Conc.												
Conc.												
Conc.												
Terp.												
Terp.	blnk	blnk	cntr.-	cntr.-	cntr.+	cntr.+						

	PS	G3	AU	PD	PC	LM	PM	PL	GA	LS	OC	GS
Terp.							blnk	blnk	cntr.-	cntr.-	cntr.+	cntr.+
Terp.												
Quin.												
Quin.												
Quin.												
Alcal.												
Alcal.												
Alcal.												

FIGURE 32 - DESIGN OF THE 96 WELL PLATES FOR THE MTT ASSAYS. SOURCE: the author (2008).

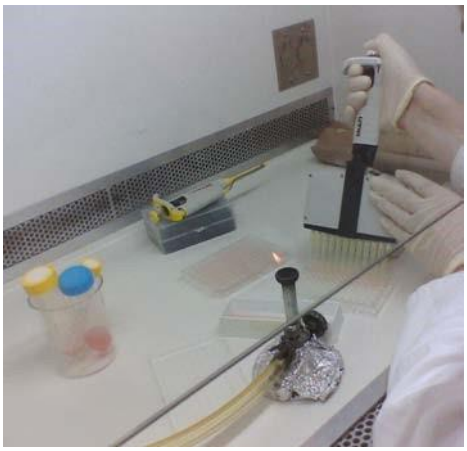


FIGURE 33 **-** *Leishmania* PRECULTURES BEING TRANSFERRED TO 96 WELL MICROPLATES WITH A MULTIVIAL MICROPIPETTE. SOURCE: the author (2008).

Plates were incubated at 25°C for 72h. Motility of the parasites was assessed periodically with an inverted optical microscope, directly on the plates. Microscope observations also served for contamination monitoring.

After this previous incubation time, 10µL of MTT reagent (10mg/mL) was added to each well. After an additional incubation time (4 hours, 25°C), 100µL of a stop solution (50% isopropanol, SDS 10mg/mL) was added to each well (FIGURE 33). Plates were then incubated at room temperature for half an hour and read with a XS Powerwave plate reader (FIGURE 34). The plate reader was programmed to agitate for 1 minute before reading. A reading wavelenght of 540nm was chosen, based on literature data and spectral scans of positive and negative controls.

FIGURE 34 - MICROPLATES AFTER INCUBATION WITH MTT REAGENT (LEFT). MICROPLATE READER COUPLED WITH A COMPUTER (RIGHT). SOURCE: the author (2008).

4.5.6 Radiolabelled thymidine method

4.5.6.1 Selected extracts

The extracts that had shown the highest activity on the previous MTT tests for each *Leishmania* species were chosen for the subsequent analysis.

Especially the concentrated and quinones extracts of some macromycetes species were considered worth for reevaluation.

Following there is a list of all the macromycetes extracts wich had shown high activities against each *Leishmania* species with the MTT methodology. These were selected for the radiolabelled thymidine assay.

a) *Leishmania enriettii*

The following extracts were selected for testing against *L. enriettii* by radiolabelled thymidine methodology:

Concentrated: *Perenniporia martiusii, Plectania* sp*., Lepista sordida, Oudemansiella canarii, Lycoperdon marginatum, Pleurotus calvescens and Pycnoporus sanguineus*.

Quinones: *Perenniporia martiusii, Plectania* sp*., Lepista sordida, Oudemansiella canarii, Lycoperdon marginatum, Pleurotus calvescens, Ganoderma stipitatum, Pleurotus djamor and Pycnoporus sanguineus*.

b) *Leishmania infantum*

The following extracts were selected for testing against *L. infantum* by radiolabelled thymidine methodology:

Concentrated: *Pleurotus calvescens, Perenniporia martiusii, Plectania* sp*., Ganoderma australe* and *Ganoderma stipitatum*.

Quinones: *Pleurotus djamor, Pleurotus calvescens, Lycoperdon marginatum, Perenniporia martiusii, Plectania* sp*., Oudemansiella canarii* and *Ganoderma stipitatum.*

c) *Leishmania braziliensis*

The following extracts were selected for testing against *L. braziliensis* by radiolabelled thymidine methodology:

Concentrated: *Perenniporia martiusii, Plectania* sp*., Ganoderma australe*, *Lycoperdon marginatum* and *Oudemansiella canarii.*

Quinones: *Perenniporia martiusii, Plectania* sp., *Lepista sordida*, *Oudemansiella canarii*, *Ganoderma stipitatum*, *Lycoperdon marginatum*, *Pleurotus calvescens* and *Pleurotus djamor.*

4.5.6.2 Radiolabelled thymidine method protocol

As for MTT tests, all *Leishmania* strains were precultured in Roux bottles, both on CCS, RPMI and on modified LIT medium, at 25°C. Samples were taken periodically from the cultures, to monitorize parasite counts and activity. Parasite countings were performed with a Thoma chamber, as described in section 4.5.4. The best medium was chosen for each *Leishmania* species, based on the parasite countings and direct observation of motility.

When *Leishmania* counts were on the 10^6~10^7 (parasites/mL) range, cultures were dilluted with the respective medium until the 10^5~10^6 (parasites/mL) range. Properly dilluted *Leishmania* cultures were distributed in 96-well microplates, 100µL per well. Then, 100µL from each of the 60 macromycete extracts were also added to each microplate well (in triplicate). Positive, negative controls and a blank were added too.

Positive control consisted of 100µL Glucantime (300mg/mL) instead of macromycete extract. Negative control consisted of 100µL of the respective culture medium instead of extracts. The blank consisted of 200µL of culture medium only, without *Leishmanias* and without the addition of the extracts.

Each well received 20µL of radiolabelled thymidine, 0,1µ*C*, dilluted in the respective culture medium (LIT for *L. enriettii* and CCS liquid for *L. infantum* and *L. braziliensis*). Thymidine was labelled with 14 carbon (^{14}C). 20µL of each radiolabelled solution were kept as a reading control.

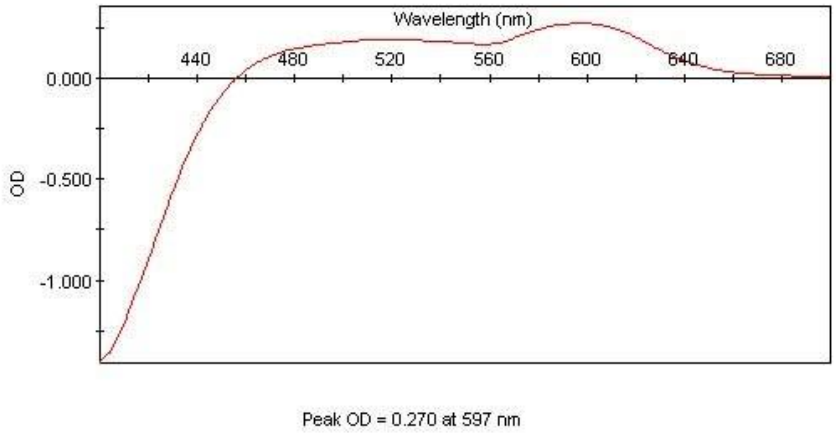
Plates were incubated for 40h at 25°C. After incubation, cells were harvested from each well and transferred to a filtering membrane, with a cell harvester. Filter pieces containing the radiolabelled cells were transferred to tubes, containing 3mL of a scintillation solution. Radioactive emission intensity of the samples were determined in a scintillation reader (FIGURE 35).

FIGURE 35 - SCINTILLATION READER USED FOR RADIOACTIVITY MEASURING OF THE RADIOLABELLED THYMIDINE SAMPLES. SOURCE: the author (2008).

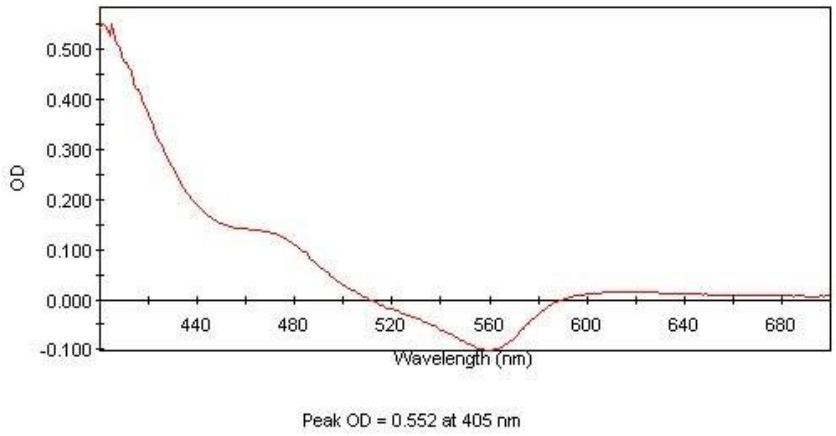
5 RESULTS

5.1 BIOACTIVITY OF EXTRACTS BY MTT METHOD

The best wavelenght range for this assay was determined by comparing the spectral scans of the positive and negative controls. Wavelenght of 540nm was chosen based on the comparative analysis of GRAPHICS 1 and 2 and literature suggestions. Medium's interference was not significative.

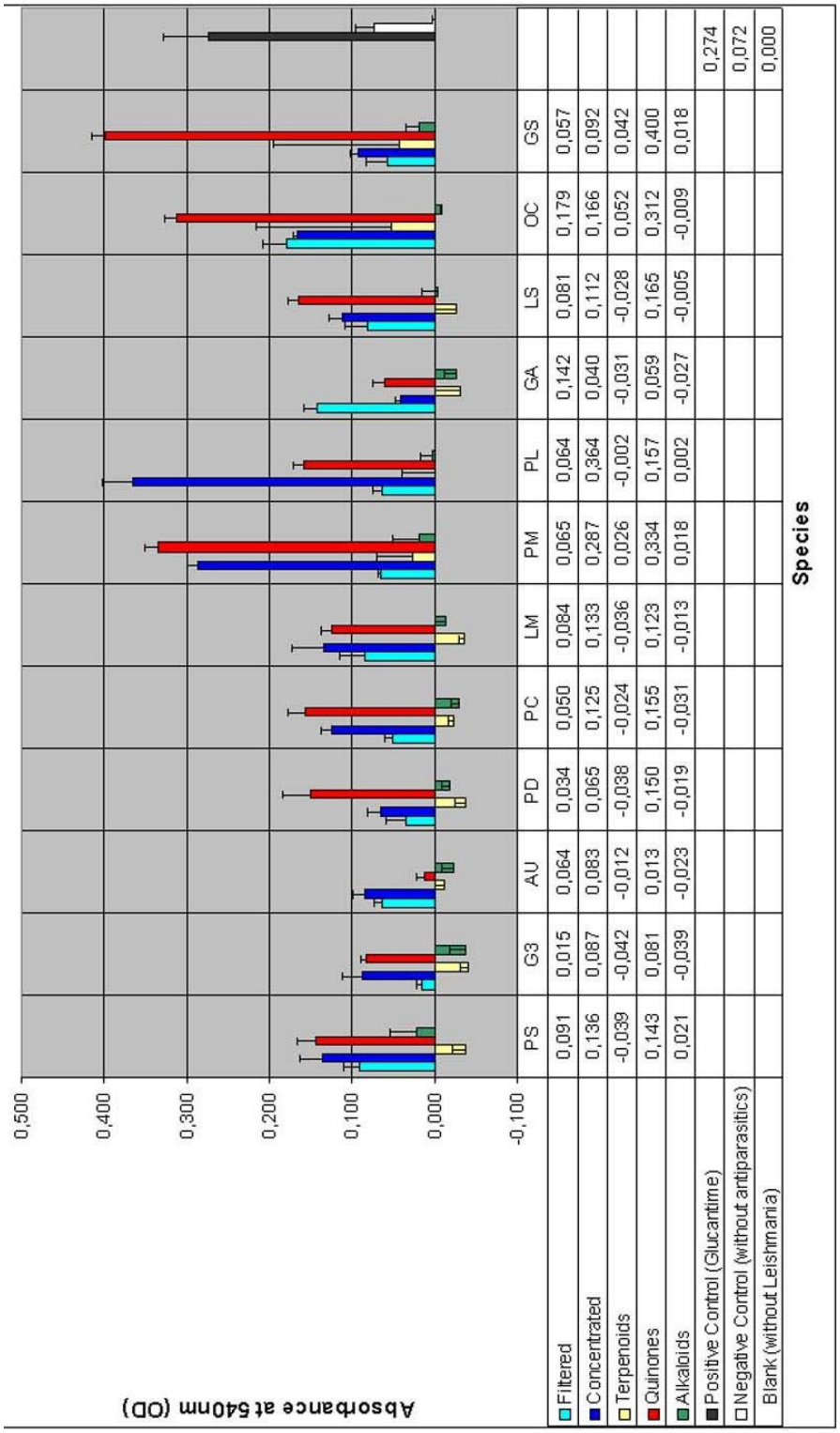


GRAPHIC 1 - SPECTRAL SCAN OF MTT POSITIVE CONTROL. SOURCE: the author (2008).

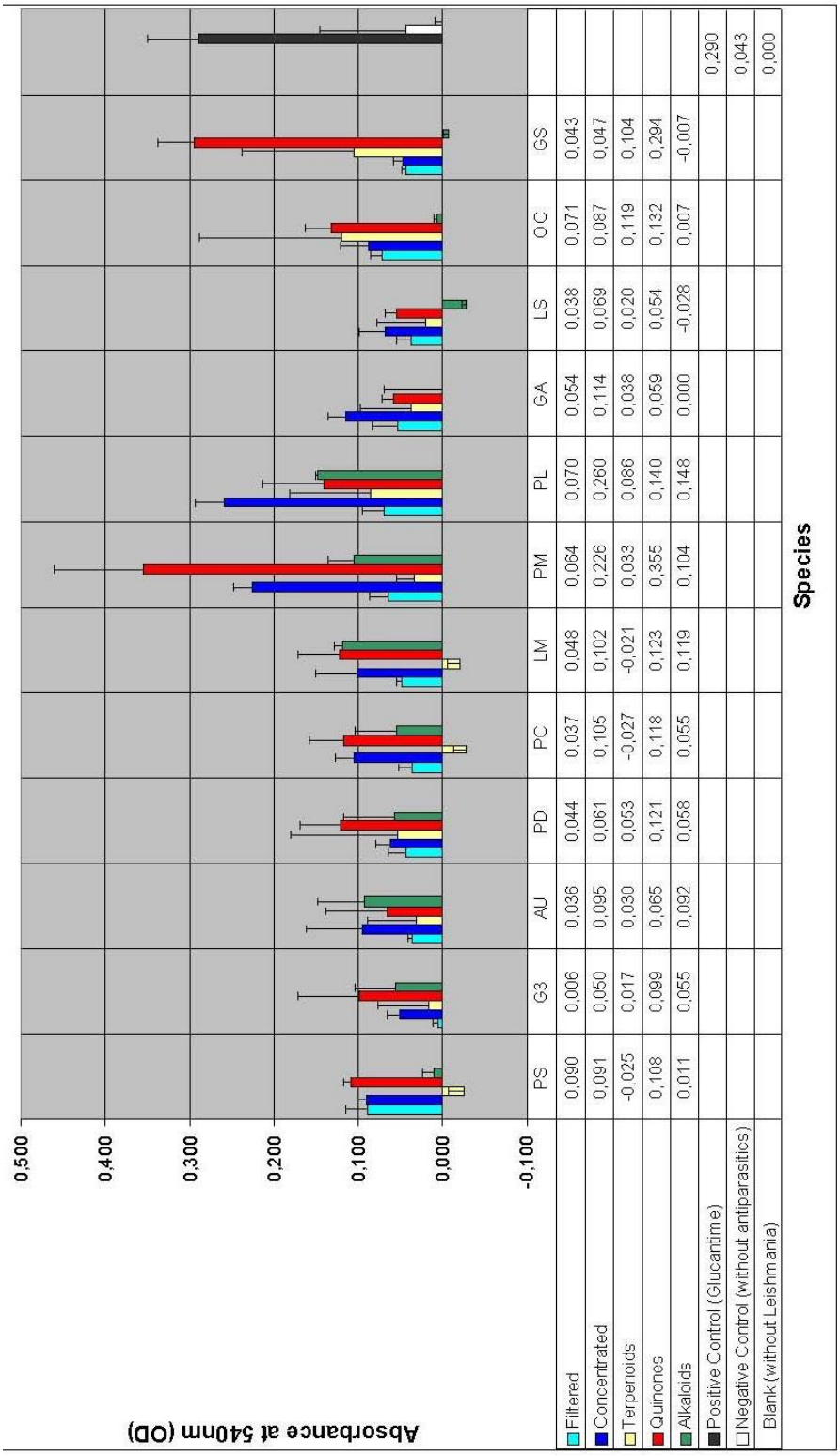


GRAPHIC 2 - SPECTRAL SCAN OF MTT NEGATIVE CONTROL. SOURCE: the author (2008).

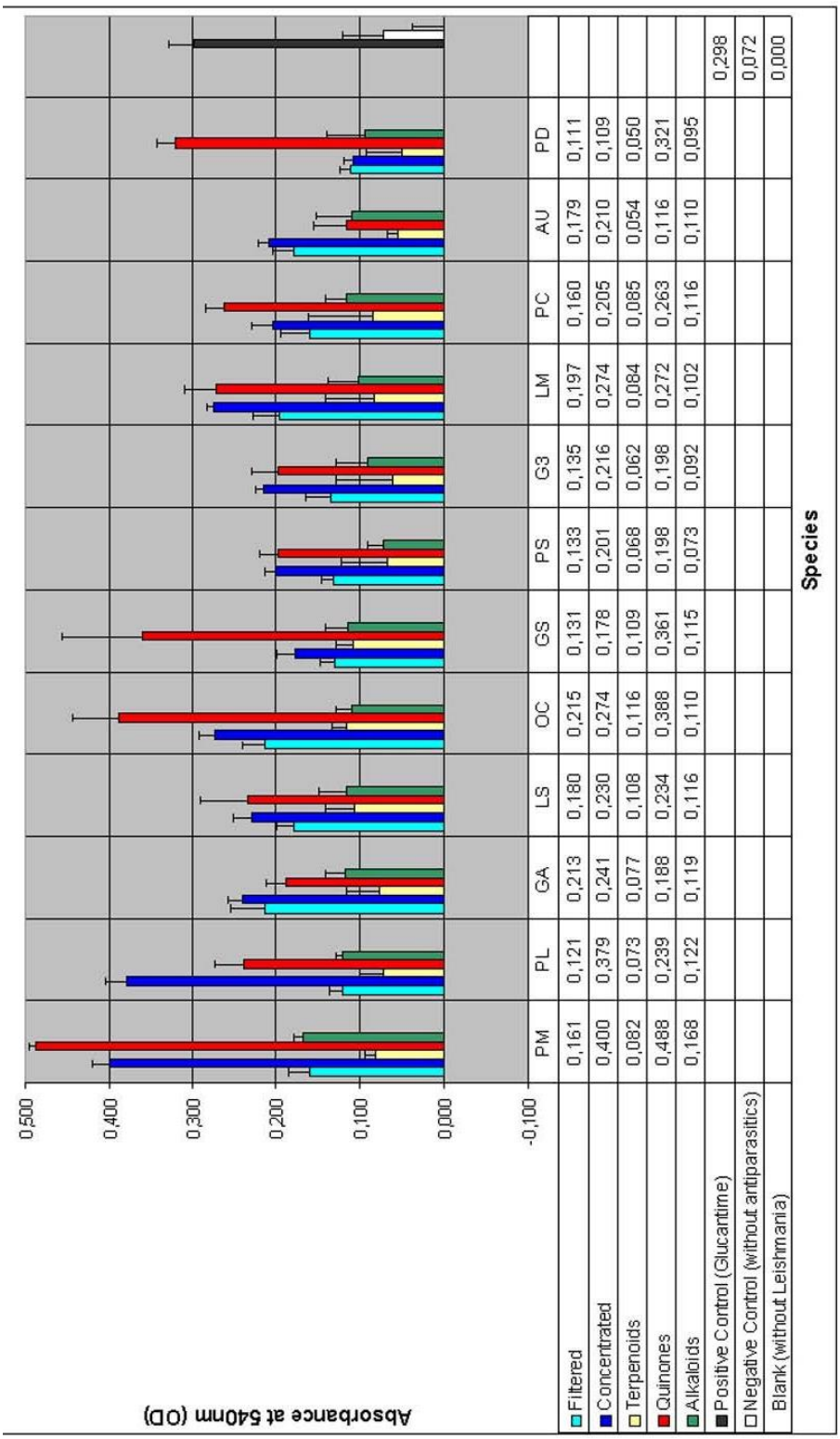
The zeroed absorbance value of the positive control was nearly 0,290 and of the negative control was about 0,062. The absorbance values for the test groups (60 different macromycete extracts) varied from -0,042 to 0,488 (GRAPHICS 3, 4 and 5).



GRAPHIC 3 – BIOACTIVITY OF 60 EXTRACTS OBTAINED FROM 12 DIFFERENT MACROMYCETES' SPECIES, AGAINST *L. enriettii*, USING MTT METHODOLOGY. PS = *Pycnoporus sanguineus*; G3 = *Ganoderma australe*; AU = *Auricularia fuscosuccinea*; PD = *Pleurotus djamor*; PC = *Pleurotus calvescens*; LM = *Lycoperdon marginatum*; PM = *Perenniporia martiusii*; PL = *Plectania* sp.; GA = *Ganoderma applanatum*; LS = *Lepista sordida*; OC = *Oudemansiella canarii*; GS = *Ganoderma stipitatum*.
SOURCE: the author (2008).



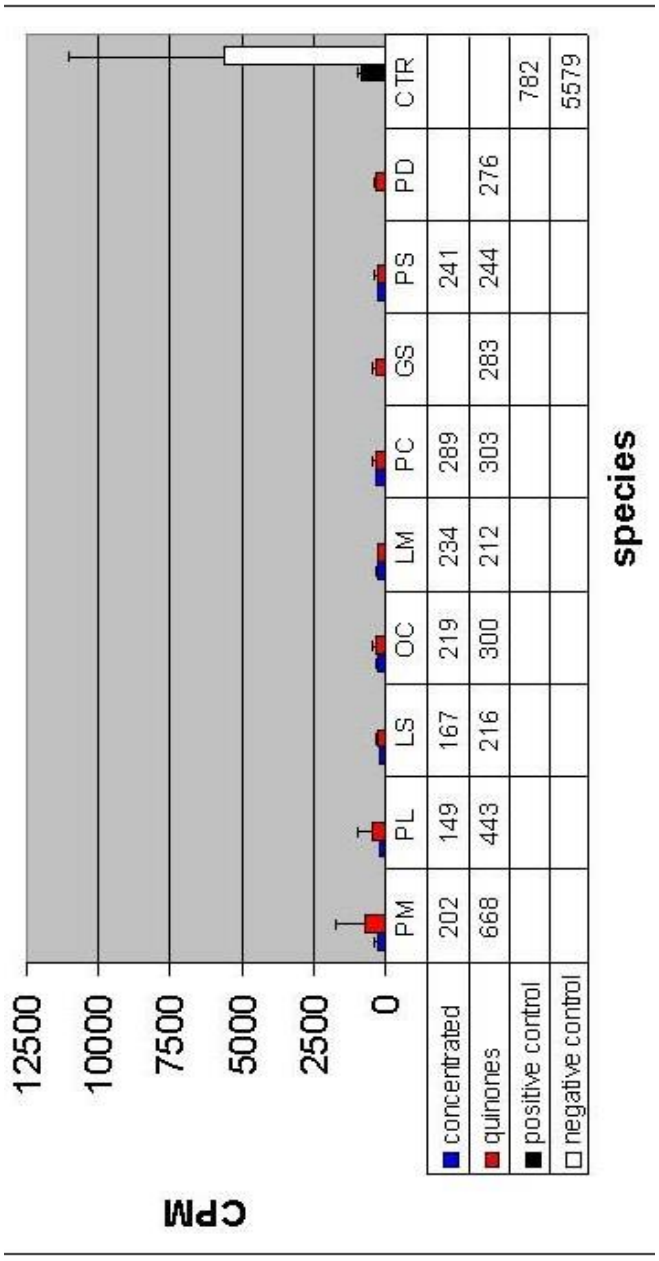
GRAPHIC 4 – BIOACTIVITY OF 60 EXTRACTS OBTAINED FROM 12 DIFFERENT MACROMYCETES' SPECIES, AGAINST *L. infantum*, USING MTT METHODOLOGY. PS = *Pycnoporus sanguineus*; G3 = *Ganoderma australe*; AU = *Auricularia fuscosuccinea*; PD = *Pleurotus djamor*; PC = *Pleurotus calvescens*; LM = *Lycoperdon marginatum*; PM = *Perenniporia martiusii*; PL = *Plectania* sp.; GA = *Ganoderma applanatum*; LS = *Lepista sordida*; OC = *Oudemansiella canarii*; GS = *Ganoderma stipitatum*.
SOURCE: the author (2008).



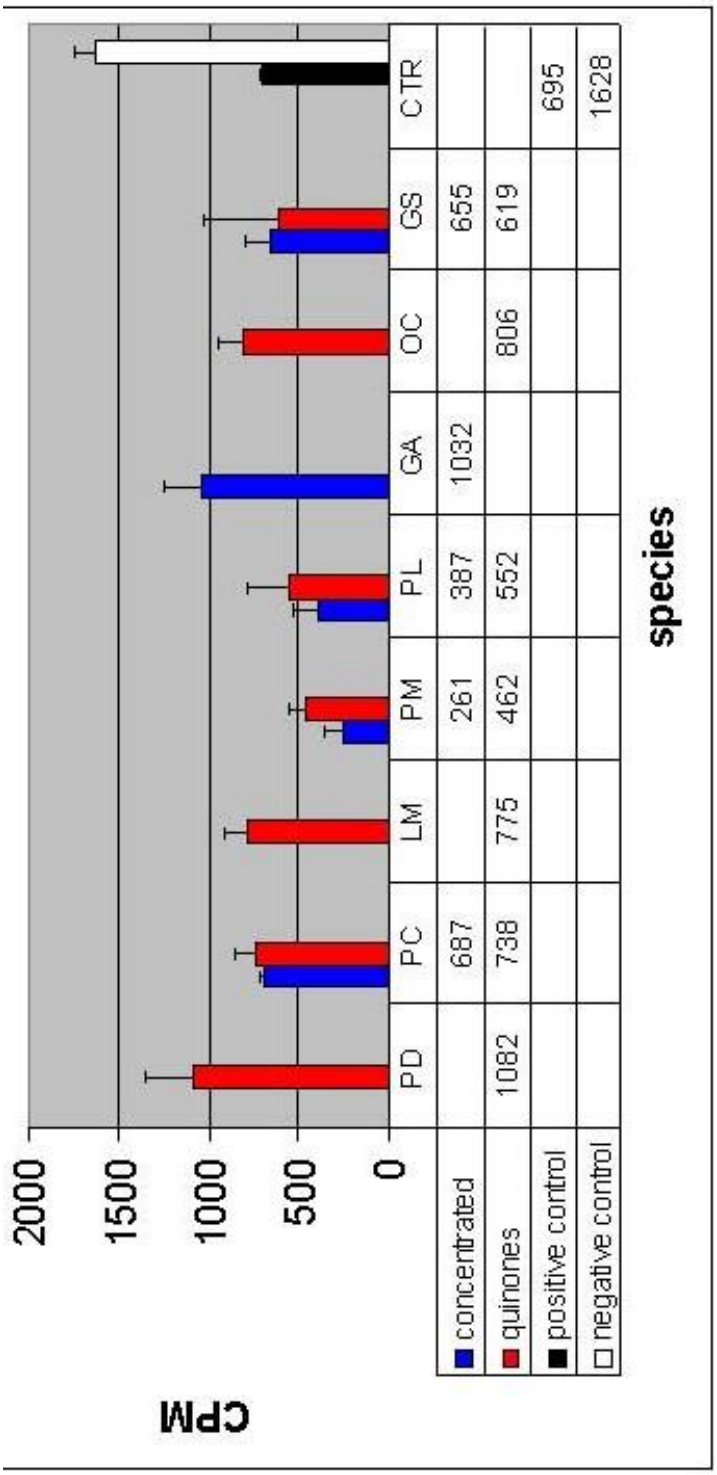
GRAPHIC 5 – BIOACTIVITY OF 60 EXTRACTS OBTAINED FROM 12 DIFFERENT MACROMYCETES' SPECIES, AGAINST *L. braziliensis*, USING MTT METHODOLOGY. PS = *Pycnoporus sanguineus*; G3 = *Ganoderma australe*; AU = *Auricularia fuscosuccinea*; PD = *Pleurotus djamor*; PC = *Pleurotus calvescens*; LM = *Lycoperdon marginatum*; PM = *Perenniporia martiusii*; PL = *Plectania* sp.; GA = *Ganoderma applanatum*; LS = *Lepista sordida*; OC = *Oudemansiella canarii*; GS = *Ganoderma stipitatum*.
SOURCE: the author (2008).

5.2 BIOACTIVITY OF EXTRACTS BY RADIOLABELLED THYMIDINE METHOD

CPM reading was approximatedly 739 for the positive controls. The negative controls mean reading was of about 3603. Test groups readings results varied from 1082 to as low as 149 (GRAPHICS 6 and 7).



GRAPHIC 6 – BIOACTIVITY OF 16 EXTRACTS OBTAINED FROM 9 DIFFERENT MACROMYCETES' SPECIES, AGAINST *L. enriettii*, USING RADIOLABELLED THYMIDINE METHODOLOGY. PM = *Perenniporia martiusii*; PL = *Plectania* sp.; LS = *Lepista sordida*; OC = *Oudemansiella canarii*; LM = *Lycoperdon marginatum*; PC = *Pleurotus calvescens*; GS = *Ganoderma stipitatum*; PS = *Pycnoporus sanguineus*; PD = *Pleurotus djamor*. SOURCE: the author (2008).



GRAPHIC 7 – BIOACTIVITY OF 12 EXTRACTS OBTAINED FROM 8 DIFFERENT MACROMYCETES' SPECIES, AGAINST *L. infantum*, USING RADIOLABELLED THYMIDINE METHODOLOGY. PD = *Pleurotus djamor*; PC = *Pleurotus calvescens*; LM = *Lycoperdon marginatum*; PM = *Perenniporia martiusii*; PL = *Plectania* sp.; GA = *Ganoderma applanatum*; OC = *Oudemansiella canarii*; GS = *Ganoderma stipitatum*. SOURCE: the author (2008).

6 DISCUSSION

All the macromycete species tested in this experiment were collected, identified and isolated during the course period. More than 30 species were successfully isolated, including: *Agaricus sylvaticus, Auricularia fuscosuccinea, Chlorophyllum molybdites, Coprinus comatus, Lepista sordida, Ganoderma applanatum, Ganoderma australe, Ganoderma stipitatum, Leucoagaricus lilacinus, Lycoperdon marginatum, Pisolythus tinctorus, Phallus merulinus, Plectania* sp*., Psylocibe cubensis, Pycnoporus sanguineus, Pleurotus calvescens Pleurotus djamor and Trametes cubensis*.

At least 15 macromycete species were successfully cultivated in the formulated liquid medium mentioned in the section 4.3 of this work. Twelve of those were processed as described throughout the section 4.4, rendering 60 different extracts. All of the 60 macromycete extracts were tested against three different species of *Leishmania*.

The first technique tried for activity evaluation of the extracts – direct observation of motility - had proven excessively subjective. As results were not on a yes/not basis, a scale of four levels of motility was applied, but even this way, results were not consistent.

As this happened, MTT methodology was chosen for trying to better quantify bioactivities of the extracts. On the first pilot experiment with MTT, it was detected that blood cells in the CCS medium interfered with it, giving positive results even for the negative control. This problem was successfully solved with the application of a cell disruption protocol, explained in 4.5.3.

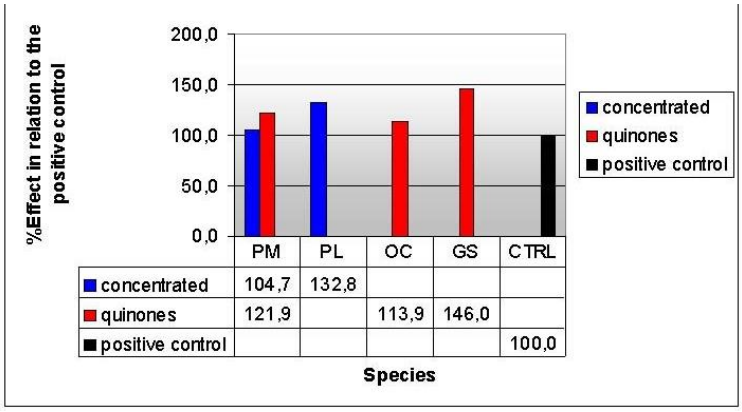
MTT results were compared by one-way-ANOVA and differences between the test groups and the positive control were determined by Dunnett´s Multi Comparison post-test for 1% and 5% significance levels. Statistical analysis of the data was performed with GraphPad Prism 3.0 software.

6.1 MTT

6.1.1 *Leishmania enriettii*

Plectania sp. concentrated and *Ganoderma stipitatum* quinones extracts were both statistically more effective than Glucantime 300mg/mL against *L. enriettii* for a 1% significance level (P<0,01). *Perenniporia martiusii* quinones extract was also more effective than the positive control for a 5% (P<0,05) significance level. *Oudemansiella canarii* quinones and *P. martiusii* concentrated extracts had as much activity as the positive control for P<0,05 (GRAPHIC 8).

The most active extract tested against *L. enriettii* was *G. stipitatum* quinones, wich showed 46% more activity than the positive control. Followed by *Plectania* sp. concentrated, almost 33% more active than Glucantime. It is interesting to note that *O. canarii*, wich is an edible species, had shown almost 14% more activity than the control.

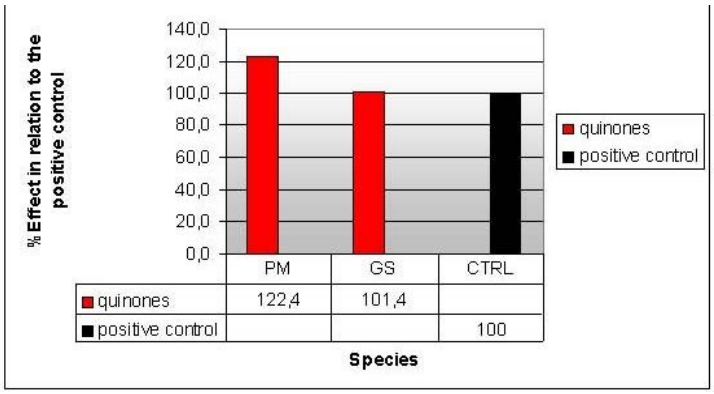


	PM	PL	OC	GS	CTRL
concentrated	104,7	132,8			
quinones	121,9		113,9	146,0	
positive control					100,0

GRAPHIC 8 - EFFECTS (IN %) OF THE MOST ACTIVE EXTRACTS TESTED AGAINST *L. enriettii* IN COMPARISON TO GLUCANTIME® 300 mg/mL (POSITIVE CONTROL), THROUGH MTT METHODOLOGY. PM = *Perenniporia martiusii*; PL = *Plectania* sp.; OC = *Oudemansiella canarii*; GS = *Ganoderma stipitatum*. SOURCE: the author (2008).

6.1.2 *Leishmania infantum*

Perenniporia martiusii and *Ganoderma stipitatum* quinones extracts were as active as the positive control for a 5% significance level (P<0,05) (GRAPHIC 9).



GRAPHIC 9 - EFFECTS (IN %) OF THE MOST ACTIVE EXTRACTS TESTED AGAINST *L. infantum* IN COMPARISON TO GLUCANTIME® 300 mg/mL (POSITIVE CTRL), THROUGH MTT METHODOLOGY. PM = *Perenniporia martiusii*; GS = *Ganoderma stipitatum*. SOURCE: the author (2008).

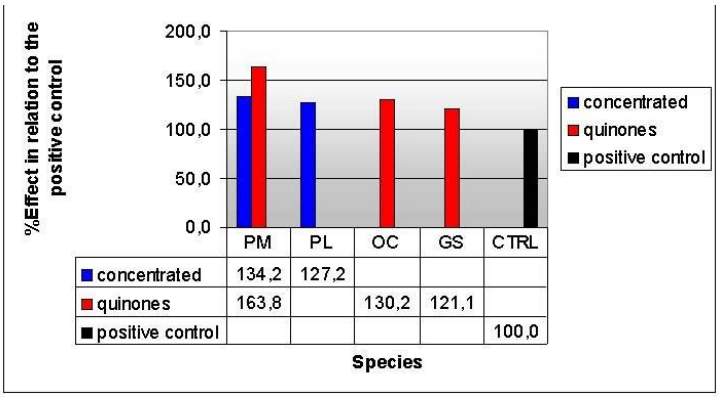
Even without showing statistical difference to the control, *P. martiusii* and *G. stipitatum* quinones extracts exhibited higher mean values for their activities than Glucantime. *P. martiusii* quinones, the most active extract tested against *L. infantum*, was 22,5% more effective than the positive control, according exclusively to the mean values.

6.1.3 *Leishmania braziliensis*

Perenniporia martiusii and *Plectania* sp. concentrated extracts were more active than the positive control for a 1% significance level (P<0,01) (GRAPHIC 10).

P. martiusii quinones extract was more effective than Glucantime 300mg/mL for a 1% significance level (P<0,01). *Ganoderma australe*, *Lycoperdon marginatum* and *Oudemansiella canarii* concentrated extracts had the same activity as the positive control for a 5% significance level (P<0,05).

Plectania sp., *Lepista sordida*, *Oudemansiella canarii*, *Ganoderma stipitatum*, *Lycoperdon marginatum*, *Pleurotus calvescens* and *Pleurotus djamor* quinones extracts exhibited as much activity as Glucantime 300mg/mL for a 5% significance level (P<0,05).



GRAPHIC 10 - EFFECTS (IN %) OF THE MOST ACTIVE EXTRACTS TESTED AGAINST *L. braziliensis* IN COMPARISON TO GLUCANTIME® 300 mg/mL (POSITIVE CONTROL), THROUGH MTT METHODOLOGY. PM = *Perenniporia martiusi*i; PL = *Plectania* sp.; OC = *Oudemansiella canarii*; GS = *Ganoderma stipitatum*. SOURCE: the author (2008).

The highest antileishmanial activity against *L. braziliensis* was observed for the *P. martiusii* quinones extract. It was almost 64% more active than Glucantime® 300mg/mL. *O. canarii* quinones showed 30,2% more activity than the positive control.

After this first screening procedure, the most well succeded extracts were reevaluated with a radiolabelled thymidine method, wich should be a more sensible bioassay, in order to confirm antiparasitic activities found.

6.2 RADIOLABELLED THYMIDINE

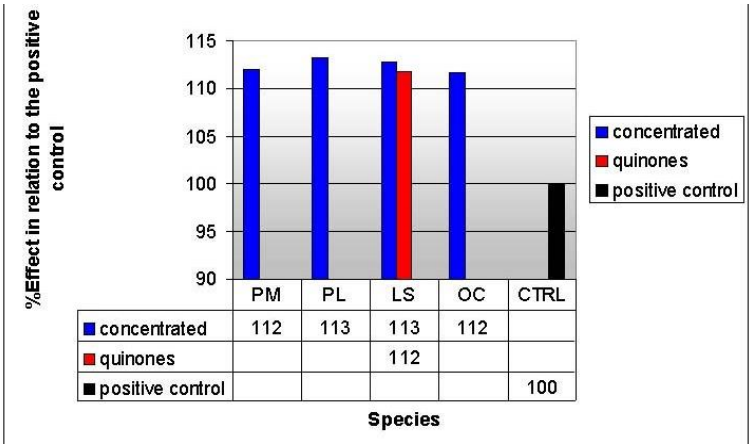
Radiolabelled thymidine results were not very accurate. Some technical and operational procedures still must be verified and perfected. The equipments must also be inspected. But even without that sharpness, radiolabelled thymidine assays results suggest a confirmation of the MTT results, pointing potential antileishmanial substances.

Results dispersion was relatively high, and some experimental points have been discarded. Results here presented represent at least duplicates.

6.2.1 *Leishmania enriettii*

All tested extracts were significantly active against *L. enriettii* when compared to the negative control, for a 1% significance level. And althogh statistical analysis point no activity difference between all the tested extracts and glucantime ($P<0,05$), all the mean

values of CPM measured for the tested substances are lower than those of the positive control, wich means they probably had more activity than Glucantime 300mg/mL. Anyway, statistical analysis don´t allow us to infer higher activity to those extracts, because the experiments results had high dispersion coefficients.



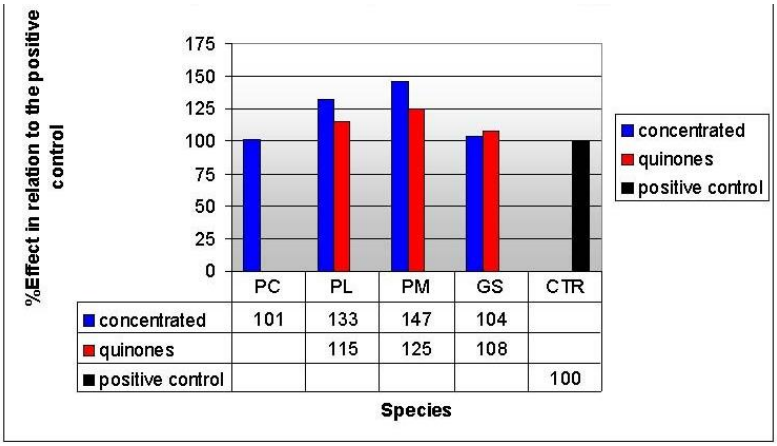
GRAPHIC 11 - EFFECTS (IN %) OF THE MOST ACTIVE EXTRACTS, TESTED AGAINST *L. enriettii* IN COMPARISON TO GLUCANTIME® 300 mg/mL (positive control), THROUGH RADOLABELLED THYMIDINE METHODOLOGY. PM = *Perenniporia martiusi*i; PL = *Plectania* sp.; LS = *Lepista sordida*; OC = *Oudemansiella canarii*. SOURCE: the author (2008).

Highest mean differences in comparison to the positive control were observed for *P. martiusii, Plectania* sp.*, L. sordida* and *O. canarii* concentrated and *L. sordida* quinones extracts. Both *L. sordida* and *O. canarii* are known to be edible. All of these extracts activity mean were about 12,5% higher than the positive control (GRAPHIC 11).

6.2.2 *Leishmania infantum*

P. martiusii concentrated extract exhibited more activity than the positive control for a 1% significance level (P<0,01). *Plectania* sp. concentrated extract was also more active than the positive control for a 5% significance level. *P. calvescens* and *G. stipitatum* concentrated extracts were statistically as active as Glucantime 300mg/mL for a 5% significance level.

All of the quinones extracts activities were no statistically different from the positive control for P<0,05. The mean CPM values for most of the tested extracts were much lower than that of the positive control, but statistical analysis don´t allow us to infer activity to those extracts, because the experiments results had high dispersion coefficients. Highest mean differences in comparison to the positive control group were observed for *P. martiusii* and *Plectania* sp. concentrated. These extracts were 47% and 33% more active than Glucantime®, respectively (GRAPHIC 12).



	PC	PL	PM	GS	CTR
concentrated	101	133	147	104	
quinones		115	125	108	
positive control					100

GRAPHIC 12 - EFFECTS (IN %) OF THE MOST ACTIVE EXTRACTS TESTED AGAINST *L. infantum* IN COMPARISON TO GLUCANTIME® 300 mg/mL (POSITIVE CONTROL), THROUGH RADOLABELLED THYMIDINE METHODOLOGY. SOURCE: the author (2008).

6.2.3 *Leishmania braziliensis*

L. braziliensis species was not evaluated by this methodology yet. After adjustment of the technique, besides repeating assays for the other two already tested species, extracts that exhibited the highest activity against *L. braziliensis* with MTT are also going to be tested with this protocol.

6.3 SUMMARY OF THE RESULTS

As there are many interesting results, the best way to visualize all of them at the same time is probably a table. All of the extracts that showed as active as or more active than the positive control (Glucantime® 300mg/mL) for both methodologies and each of the leishmania species are listed in TABLE 7.

TABLE 7 – SUMMARY OF THE MOST INTERESTING RESULTS. EXTRACTS ARE REPRESENTED BY A THREE LETTERS CODE. THE FIRST TWO CORRESPOND TO MACROMYCETE SPECIES (ABBREVIATIONS LIST) AND THE THIRD DENOTES THE EXTRACTIVE PROCEDURE: F - FILTERED, C - CONCENTRATED, T - TERPENES, Q - QUINONES, A – ALKALOIDS.

Leishmania SPECIES / METHODOLOGY	EXTRACTS MORE ACTIVE THAN THE POSITIVE CONTROL	EXTRACTS AS ACTIVE AS THE POSITIVE CONTROL
L. enriettii / MTT	PLC, GSQ, PMQ	OCQ, PMC
L. enriettii / radiolabelled thymidine		PMC, PLC, LSC, OCC, LMC, PCC, PSC, PMQ, PLQ, LSQ, OCQ, LMQ, PCQ, GSQ, PDQ, PSQ
L. infantum / MTT		PMQ, GSQ
L. infantum / radiolabelled thymidine	PLC, PMC	PCC, GSC, PDQ, PCQ, LMQ, PMQ, PLQ, OCQ, GSQ
L. braziliensis / MTT	PLC, PMC, PMQ	G3C, LMC, OCC, PLQ, LSQ, OCQ, GSQ, LMQ, PCQ, PDQ
L.braziliensis / radiolabelled thymidine		

SOURCE: The author (2008).

7 CONCLUSION

Expressive activities were found on at least nineteen extracts, even in comparison to Glucantime 300mg/mL. Four extracts had shown even more activity than the positive control. Three against *L. enriettii,* three against *L. braziliensis* and two against *L. infantum*. Nineteen extracts were found to be as active as the positive control. Nine of wich against *L. infantum,* sixteen against *L. enriettii* and ten against *L. braziliensis*.

P. martiusii quinones extract showed almost 64% more activity than the positive control against *L. braziliensis*, with the MTT methodology.

G. stipitatum quinones, showed 46% more activity than the positive control against *L. enriettii*, with the MTT methodology.

Although not showing statistical difference to the positive control, due to the dispersion of the data, *P. martiusii* quinones extract mean activity, was 22,5% higher than the positive control against *L. infantum*, with MTT.

Highest effects against *L. infantum* were observed, with the radiolabelled thymidine methodology, for *P. martiusii* and *Plectania* sp. concentrated. These extracts were 47% and 33% more active than Glucantime® 300mg/mL, respectively.

The most active substances tested were mainly quinones, in agreement with previous literature information. With a few exceptions, quinones extracts were significantly more active against all *Leishmania* species than any other extract types. And although some quinones extracts had shown high activity levels, many others didn´t affect leishmanias, indicating that the extractive procedure itself didn´t interfere with the results.

Concentrated extracts also showed some good results. There was some correlation between mushroom species and antileishmanial activity. That is, when a quinones extract from a mushroom species have great activity, it is very likely that the concentrated extract from the same species also have.

As relatively high temperatures were used in the downstream processes (for concentration and solvent evaporation), active substances are thermostable. This can save processment time, and make storage and transport cheaper. This also secures substances will resist high temperature procedures, as pasteurization for example.

Some extracts had shown results lower than the negative control. This suggests those extracts serve as culture medium or culture medium complement for leishmanias and/or contain some compound(s) that stimulate their proliferation. The possibility those extracts interfered with the MTT procedure was discarded by direct observation with an inverted microscope.

It is interesting to note that at least five active species are known to be edible. Those are surely functional food candidates, that must be assayed through the proper means to scientifically assess their potential when consumed orally, as diet supplementation bioproducts.

The most promisor mushrooms species suggested by this experiment, as a source of antileishmanial compounds are *Plectania* sp*., Ganoderma stipitatum*, *Perenniporia martiusii* and *Oudemansiella canarii.* Concentrated and quinones preparations were the most active.

This experiment was designed as a mean to evaluate a great number of preparations for their antiparasitic activity. The adopted methodologies were successful in being sufficiently fast, practical and accurate, to point potential new antiparasitic substances for further studies.

8 PERSPECTIVES

This experiment was the first step of an antileishmanial bioproduct development process. As some of the tested extracts showed really high activities in this screening, these are candidates for subsequent research.

We must have in mind this was a direct toxicity *in vitro* evaluation. Compounds that didn´t show direct activity shouldn´t be totally discarded as antiparasitic agents. There is still the possibility that these compounds can exhibit indirect activity against parasites, through immune system modulation. Biological Response Modifiers activities can be tested over immune cells cultures or through *in vivo* experiments.

Compounds that showed a high antiparasitic activity in this study should be further tested by other complementar *in vitro* methodologies, and even through *in vivo* assessments and clinical trials. These active extracts must be tested for toxicity against human cells and experimental animals before they are clinically tested. As pentavalent antimonials are aggressive, it is interesting to further study even the extracts with as much activity as Glucantime®, because they can possibly show less side effects.

Active extracts must be further fractioned in order to determine the active principles responsible for the antileishmanial effects observed. This can suggest molecular groups for further antileishmanial biocompounds screening. Characterization of these molecules and their mechanisms of action can also offer insights useful for antileishmanial drug design.

As active principles are identified, parameters, as process duration, aeration, agitation, temperature, pH, culture medium composition and extractive procedures can be optimized for maximizing active biomolecules production and recovery.

The effect of the bioactive compounds can be improved by chemical manipulations of the molecules, such as complexation with vanadium ions, for example. Combinations of active substances must be considered too.

Edible active mushrooms should be analysed through other methodologies, in order to evaluate their potential as antiparasitic nutraceuticals. The edibility is an indicative their compounds won´t show significative toxicity against human cells. This is important, as specificity is probably the main goal of drug design, besides effectiveness.

Infinite other biomolecules are available for antiparasitic substances screening. The methodologies here described are ready for evaluating other mushroom species and other parasites. In addition, the mushroom extracts here described can be tested for other bioactivities, as against other parasites or tumors. Many possibilities have been opened.

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