ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

MOLECULAR ANALYSIS OF *LEISHMANIA* RNA VIRUS (LRV)-POSITIVE AND NEGATIVE *LEISHMANIA MAJOR* ISOLATES AND ASSESSMENT OF THE EFFICACY OF FUNGAL METABOLITES COMPARED WITH MEGLUMINE ANTIMONIATE

BUSE KAYMAZ

M.Sc. THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

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DECLARATION

I declare that this thesis work is my own work. I had no unethical behaviour at any stages from the planning to the writing of the thesis. I obtained all the information in this thesis in accordance with academic and ethical rules. I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

02.06.2023

Buse KAYMAZ

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LIST OF ABBREVIATIONS AND SYMBOLS

AMM	*Aspergillus* Minimal Liquid Medium
AMP	Antimicrobial Peptide
BC	Before Chris
BCA	Bicinchoninic Acid
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
CL	Cutaneous leishmaniasis
CPA	Cysteine protease A
CT	Computed Tomography
Cys	Cysteine
DAT	Direct Agglutination Test
DF	Dilution Factor
DHFR	Dihydrofolate Reductase-thymidylate Synthase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dsDNA	Double Strand RNA
ELISA	Enzyme-Linked Immunosorbent Test
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
GP63	Glycoprotein 63 – Leishmanolysin
HIV	Human Immunodeficiency Virus
IC50	Half maximal inhibitory concentration
IFAT	Immunofluorescent Antibody Test
IFN-γ	Interferon-gamma
kDa	KiloDalton
kDNA	Kinetoplast DNA
KMP11	Kinetoplastid Membrane Protein 11
LPG	Lipophosphoglycan
LRV	*Leishmania RNA Virus*

LRV-	*Leishmania* without *Leishmania* RNA *Virus*
LRV+	*Leishmania* with *Leishmania* RNA Virus
MCL	Mucocutaneous Leishmaniasis
MEA	Malt Extact Agar
mRNA	Messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
NCBI	National Center Biotechnology Information
NIH	National Library of Medicine
OD	Optical Density
PAHO	Pan American Health Organization
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PKDL	Post-kala-azar Dermal Leishmaniasis
RDRP	RNA-dependent-RNA Polymerase
RDT	Rapid Diagnostic Test
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute
RT-PCR	Real-Time Polymerase Chain Reaction
SF	Physiological Saline Solution
Spp.	Species
TFA	Trifluoroacetic Acid
TLR	Toll-Like Receptor
TRYR	Trypanothione Reductase
TUCIM	TU Collection of Industrial Microorganisms
VL	Visceral Leishmaniasis
WHO	World Health Organization
WS	Working Solution
WST	Water-soluble tTtrazolium Salt
XTT	Methoxynitrosulfophenyl-Tetrazolium Carboxanilide

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ÖZET

Leishmania RNA Virüsü (LRV)-Pozitif ve Negatif _Leishmania Major_ İzolatlarının Moleküler Analizi ve Meglumin Antimonat ile Karşılaştırıldığında Mantar Metabolitlerin Etkinliğinin Değerlendirilmesi

Leishmaniasis bugün dünyada, Türkiye dahil, 98 ülkede yaygın olan vektör kaynaklı bir paraziter enfeksiyon olup her yıl 1 milyondan fazla kişiyi enfekte etmektedir. Günümüzde kullanılan ilaçlara karşı direnç gelişmiş olması leishmaniasis için ciddi bir sorundur ve yaygın olarak ilaç geliştirme çalışmalarını teşvik etmektedir. *Leishmania major*, bu enfeksiyonun etkenlerinden biri olup büyük çaplı, ciddi deri enfeksiyonlarına yol açmaktadır. Önceki çalışmalarda, *L. major* içinde bulunabilen *Leishmania* RNA virüsünün (LRV) deri enfeksiyonunun klinik tablosu ve prognozunu kötüleştirebildiği bildirilmiştir; bununla birlikte LRV-*L.major* etkileşimiyle ilgili moleküler mekanizmalar henüz bilinmemektedir. Bu çalışmada, in vitro koşullarda LRV-pozitif ve negatif *L. major* izolatlarında moleküler patolojik mekanizmaların araştırılması, ayrıca bazı fungal izolatların anti-leishmanial etkinliklerinin in vitro incelenmesi amaçlanmıştır. DHFR gen ifadesinde LRV-pozitif izolatında LRV-negatife göre 3 kat daha yüksek artış gözlemlenmiştir. GP63 ve TRYR genlerinin ifadelerinde genel olarak azalış gözlemlenirken LRV-negatif izolatı LRV-pozitife göre sırasıyla 3,3 ve 0,1 oranla 6 kat daha fazla artmıştır. Dört *Aspergillus* sp. izolatı ile henüz tanımlanmamış bir yerli bir mantar örneği malt ekstresi ile *Aspergillus* minimal besiyerlerinde kültive edilmiş ve izolatların anti-leishmanial etkinlikleri, günümüzde leishmaniasis tedavisinde kullanılan ilaç olan meglümin antimonat ile karşılaştırılmıştır. Sonuçlara göre, "*Aspergillus* 2874" ve "*Aspergillus* 3508" kodlu izolatların meglümin antimonata kıyasla daha yüksek anti-leishmanial etki ve "Aspergillus 3508" dışındaki izolatlardaki ClassI hidrofobinlerin LRV-pozitif *L. major*'de antiviral etkinlik gösterdikleri belirlenmiştir. Bu ön bulgular, çalışmada incelenen mantar metabolitlerinin anti-leishmanial ve anti-LRV etki potansiyeline sahip olduklarını göstermiş olup yeni çalışmalara olanak yaratmaktadır.

Anahtar Sözcükler: *Leishmania*, İlaç, *Leishmania* RNA virüsü, *Aspergillus*, Türkiye

ABSTRACT

Molecular Analysis of *Leishmania* RNA Virus (LRV)-Positive and Negative *Leishmania Major* Isolates and Assessment of The Efficacy f Fungal Metabolites Compared with Meglumine Antimoniate

Leishmaniasis is a vector-borne parasitic disease that is prevalent in 98 countries today, including Türkiye, and infect more than a million people worldwide, annually. Resistance to current treatment options of leishmaniasis is a serious concern that encourages common drug development studies. *Leishmania major* is one of its causative agents associated with cutaneous infections that may end up with large, serious skin lesions. Previous reports suggest that *Leishmania* RNA Virus (LRV) present in *L. major* may worsen the clinical manifestations and prognosis of cutaneous leishmaniasis; however molecular mechanisms associated with LRV-L. major interactions are yet to be unveiled. In the present study, we aimed both to assess the roles of molecular pathogenic mechanisms of LRV-positive and LRV-negative *L. major* infections in vitro, and to assess anti-leishmanial efficacies of some fungal isolates in vitro. The expression of the DHFR gene showed a 3-fold increase in LRV-positive isolates compared to LRV-negative isolates. While a general decrease in the expressions of both GP63 and TRYR genes were observed, the LRV-negative isolate exhibited 3.3-fold and 0.16-fold increase compared to the LRV-positive isolate, respectively. Isolates of four *Aspergillus sp.* and an undefined local fungus were cultivated in malt extract and aspergillus minimal media, and anti-leishmanial efficacies of their isolates were compared with meglumine antimonate, the current drug of choice for leishmaniasis treatment, in vitro. Results indicated that isolates encoded as "*Aspergillus* 2874" and "*Aspergillus* 3508" showed higher efficacy in general, than meglumine antimonate. In addition, class I hydrophobins of all fungal isolates, excluding "*Aspergillus* 3508", showed anti-viral efficacy in LRV-positive *L. major* isolate. These initial findings indicate the presence of fungal metabolites with anti-leishmanial and anti-LRV efficacies, which deserves further assessments.

Keywords: *Leishmania*, Therapeutics, *Leishmania RNA virus*, *Aspergillus*, Turkey

1. INTRODUCTION AND AIM

Leishmania is a protozoan parasite that causes leishmaniasis, a disease transmitted by the vectors *Phlebotomus spp.* and *Lutzomyia spp*., particularly in tropical and hot regions. It is considered one of the most common neglected tropical diseases, with approximately 1 million new cases reported each year, following malaria. The treatment of leishmaniasis involves the use of drugs such as pentavalent antimonials, miltefosine, pentamidine, and amphotericin B. These drugs exert their effects through various mechanisms, including cell membrane disruption, apoptosis, alteration of mitochondria, immunomodulatory functions, and DNA damage, all of which interfere with the vital functions of *Leishmania*. However, these drugs have significant side effects, with miltefosine exhibiting teratogenicity as the most serious one. Recently, the emergence of drug resistant *Leishmania* strains has highlighted the need for new treatment options.

Plants and fungi are among the potential sources of natural drugs developed against diseases. Fungi and protozoa have a distinct cell structure from mammalian cells, characterized by the presence of ergosterol. When developing a drug, it is essential to consider a pathway that does not harm the host and targets a pathway absent in the host's system. Fungi, including *Aspergillus* species, represent potential candidates for the treatment of leishmaniasis.

Aspergillus species belong to the filamentous fungi in the Ascomycetes class. The survival of fungi for thousands of years is attributed to their evolutionary mechanisms and effective self-protection strategies. They produce small rich-cysteine peptides that form rodlets, creating a hydrophobic environment and taking advantage of infection, dissemination, and evasion of host cell detection. *Aspergillus* species are widely used in the industry for enzyme production due to their high primary and secondary metabolite production capacity. However, their use in the field of health is limited. The choice of *Aspergillus* species in this study is justified by the similarities between antimicrobial peptides (AMPs) produced by *Aspergillus* and the drugs used in leishmaniasis treatment, both in terms of structural features and target regions.

This study will include a total of five samples, four of which are *Aspergillus* species collected from the rainforests of Borneo and stored by the TU Collection of Industrial Microorganisms (TUCIM). These species are numbered as 2874, 3508, 3616, and 3766. The fifth sample, Uşak6, was obtained from the fieldwork conducted by Assoc. Prof. Günseli BAYRAM AKÇAPINAR, and its precise species identification has not been determined yet. Three different methods will be employed to treat the Leishmania isolates with these samples. The first method aims to dissolve Hydrophobin Class I peptides using Trifluoroacetic Acid. The other methods involve testing the effects of different metabolites produced by the samples in different culture media, namely malt extract and *Aspergillus* minimal medium, on Leishmania isolates.

The experiment includes testing a *Leishmania major* isolate with *leishmania RNA virus* (LRV+) and another *L. major* strain without the virus (LRV-) as a positive control for meglumine antimoniate. The primary objective of this thesis is to provide a comparative evaluation of dose-response studies and molecular analyses of meglumine antimoniate in LRV+ and LRV- isolates. The investigation of the hypothesis linking the inhibition of the TRYR (Trypanothione reductase) gene to meglumine antimoniate will also be a significant contribution. Additionally, the study will examine the GP63 gene (a zinc metalloprotease or leishmanolysin) as a virulence factor and the DHFR gene (Dihydrofolate reductase) from the folate metabolic pathway, which is essential for trypanosomatids. Additionally, the fungi samples will be tested as planned. After completing the total protein concentration test for the collected samples, they will be treated with *Leishmania* cultures at various concentrations. Based on the obtained results, an inhibition trend will be identified, and the half maximal inhibitory concentration (IC50) values will be calculated. The samples showing efficacy will be further investigated for their active compounds and evaluated for cytotoxicity and other related factors in the next stage of the study.

2. BACKGROUND

2.1. Leishmaniasis

Leishmaniasis, a vector-borne disease caused by the obligate intracellular protozoan *Leishmania* species, is transmitted through the bites of blood-sucking *Phlebotomus* species in Old World and *Lutzomyia* species in New World, which are popularly known as the sandfly. *Phlebotomus* sandflies comprise over 90 species. However, it's only the infected female phlebotomine sandflies that can transmit the disease as shown Figure 1 (1).

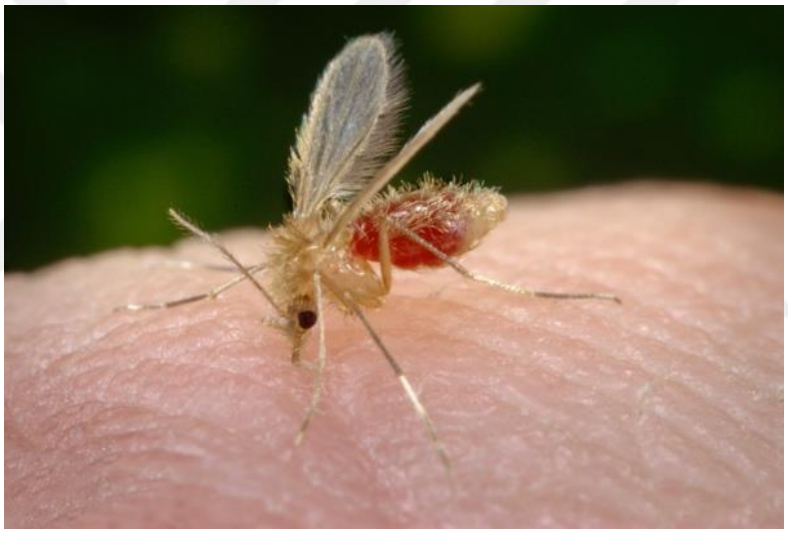

Figure 1. *Phlebotomus spp.*, commonly known as sand flies, belongs to the dipteran family Psychodidae and the subfamily Phlebotominae. The sandfly in the photo above recently completed its blood meal, which could be observed through its swollen transparent abdomen (2).

Leishmaniasis is commonly seen in tropical and subtropical regions of Africa, Asia, and South America. It is reported to the World Health Organization (WHO) from all continents except Australia and Antarctica. Approximately 200 million individuals reside in areas where leishmaniasis is endemic, and there are an estimated 4 to 12 million infected people across 98 countries (3). Each year, a significant number of new cases are reported, ranging from 0.7 to 1 million, resulting in 20,000 to 50,000 deaths annually (1).

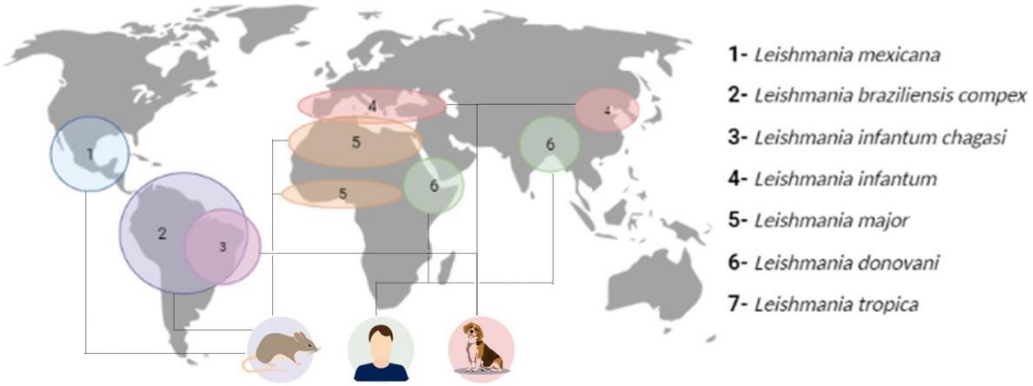

Figure 2. World map illustrating the reservoirs of Leishmania species. Created in Biorender.com and adapted from Ref (4).

Leishmaniasis is predominantly a zoonotic disease, except for *L. donovani* and *L. tropica* in Figure 2, although there is some evidence that suggests the presence of animal reservoirs for these species as well (5). There are over 53 known species of *Leishmania*, with more than 20 species capable of causing illness in humans (5). Each distinct species leads to various clinical manifestations, ranging from self-resolving cutaneous ulcers to disfiguring mucocutaneous lesions, and even life-threatening systemic visceral disease. The outcome of the disease is influenced by multiple factors, including the characteristics of the parasite, the vector responsible for transmission, and the host's immune status.

The World Health Organization (WHO) recognizes leishmaniasis as both a neglected tropical disease and a significant public health concern, necessitating the development of efficient treatment strategies and comprehensive plans for prevention and control to achieve its elimination.

2.1.1. Epidemiology

Among the 200 countries reporting to the World Health Organization, leishmaniasis is considered endemic in 98 countries (1). It may be seen sightings range from the rainforests of Central and South America to the deserts of West Asia and the

Middle East. It has not been seen so far in Australia and Antarctica. While this disease is more common in rural areas than in cities, it can also be seen in poor neighbourhoods of cities.

Approximately 12 million people have symptomatic infection and at least 120 million people are asymptomatic in many parts of the world. In addition, 1.7 billion people all over the world are at risk. According to the latest data, approximately 700,000 to 1 million new cases occur each year (1), with few of them develop to symptomatic disease. In the Old World (the Eastern Hemisphere), leishmaniasis is found in parts of the Middle East, tropical regions of Africa and Southern Europe, and in the New World (the Western Hemisphere) it is identified in both Central and South America (6).

Visceral leishmaniasis is seen in Brazil, Ethiopia, India, Somalia, and Sudan, while cutaneous leishmaniasis is more common in Afghanistan, Algeria, Brazil, Colombia, and Iran (Figure 3). Mucocutaneous leishmaniasis is mostly seen in Bolivia, Brazil, and Peru. While the incidence of visceral leishmaniasis has dropped below 100,000, the incidence of cutaneous leishmaniasis varies between 700,000 and 1.2 million (6).

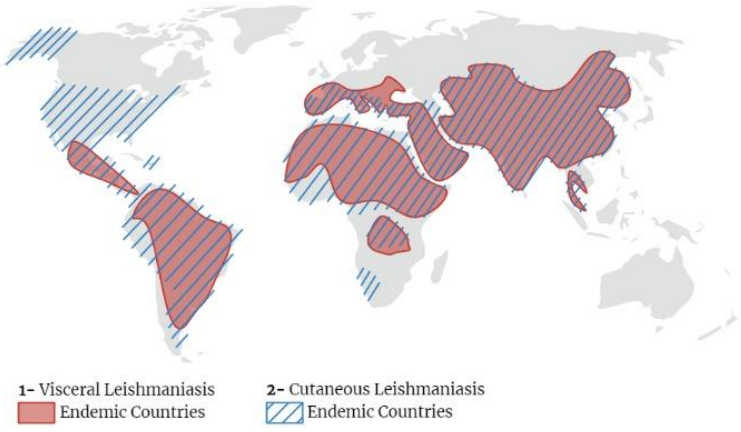

Figure 3. Status of visceral and cutaneous leishmaniasis in the World. Created in Biorender.com and adapted from Ref (7).

While leishmaniasis is more prevalent in southern hemisphere, environmental conditions for vector and reservoir species have been changing due to climate change in northern hemisphere as well, which may directly influence its prevalence in near future (8).

2.1.2. Major risk factors

Leishmaniasis is classified in the neglected tropical disease category due to the lack of financial resources in the poorest regions of the world where there are individuals who are malnourished, have poor housing conditions and no access to clean water and therefore have weakened immune systems (1,6). The inadequacy of socioeconomic conditions, especially housing and domestic health conditions, increases the risk of leishmaniasis. The lack of waste management, the presence of open sewers, and the proximity of sandfly breeding areas to human-dwelling areas can increase the risk. In case of insufficient intake of protein, iron, vitamin A and zinc in food, the risk of infections turning into diseases increases. Due to population mobility, people who are not immune to leishmaniasis and not taking precautions are more prone to infections in endemic areas. In addition, factors such as drought, flood, and famine brought about by climate change, force people to move to prevalent regions for leishmaniasis, while deforestation causes the sandfly population to increase in urbanized areas and the incidence of leishmaniasis is affected by human influx (6).

2.1.3. Clinical manifestations

Main clinical manifestations of leishmaniasis are visceral, cutaneous, and mucocutaneous. Cutaneous leishmaniasis may further appear less commonly as ulcerated, disseminated, diffuse or atypical clinical manifestations (Figure 4).

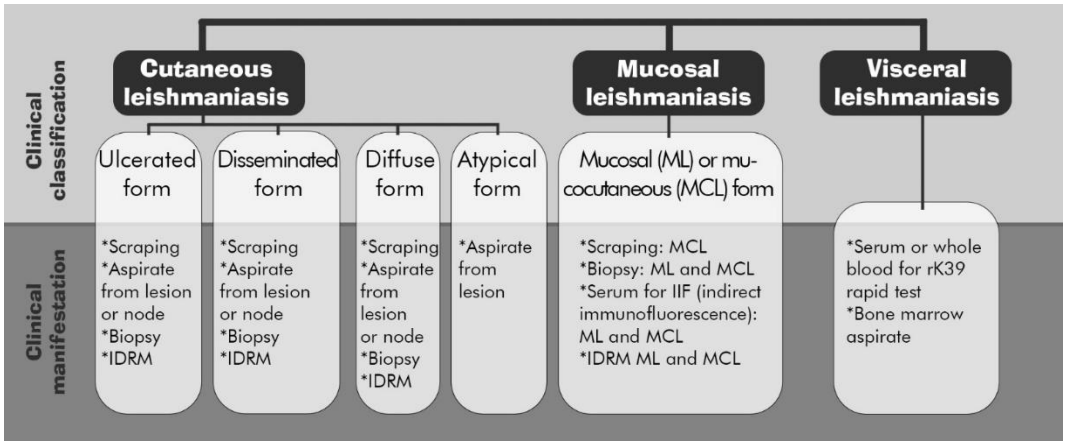

Figure 4. Classification for clinical diagnosis of leishmaniasis. Created in Biorender.com and adapted from Ref. (PAHO/WHO, 2018) (9).

Visceral leishmaniasis (VL) affects internal organs such as the spleen and liver, while cutaneous leishmaniasis (CL) causes lesions or ulcers on the skin. VL is fatal if left untreated in more than 95% of the cases. Fever, weight loss, enlarged spleen and liver, and anaemia are common symptoms. An estimated 50-90,000 new cases of VL occur each year, with most occurring in East Africa, Brazil, and India (10).

CL is the most common form of leishmaniasis. It causes permanent scars and serious disability due to ulcers and lesions on the skin. About 95% of the cases occur in the Americas, the Mediterranean basin, the Middle East, and Central Asia, with an estimated 600,000 to 1 million new cases occurring worldwide each year. Mucocutaneous leishmaniasis causes destruction of the mucous membrane of the nose, mouth, and throat. Worldwide, over 90% of this is seen in Bolivia, Brazil, Ethiopia, and Peru (1).

Except for the main clinical manifestations, post-kala-azar dermal leishmaniasis (PKDL) manifests as a papular or nodular rash that occurs in the continuation of visceral leishmaniasis, usually on the face, including the upper body and arms. While it has been observed that VL patients occur between 5-10% in Sudan, it has been reported to occur in the Indian Subcontinent. It has been observed that it mostly occurs 6 months to 1 year after the recovery of VL. The most important point about this manifestation is that individuals with PKDL are considered as a potential source of *Leishmania* infection (11).

Another situation is that HIV-positive individuals may contract leishmaniasis more commonly. While mortality rates are higher, antiretroviral treatments slow down the development of the disease and increase survival. In 2021, the presence of *Leishmania*-HIV coinfection was reported to WHO in 45 countries. In 2022, the World Health Organization presented new treatment recommendations for these patients (1).

2.1.4. Diagnosis

Various methods are available to diagnose leishmaniasis, from clinical evaluation to laboratory tests. First, clinical symptoms and travel history are assessed in the clinical evaluation. Diagnosis may be based on clinical symptoms such as skin lesions, fever, hepatosplenomegaly (enlarged liver and spleen), and lymphadenopathy (enlarged lymph nodes). In addition, the travel information of the patient in the travel history to endemic regions is crucial for the diagnosis (12).

Secondly, laboratory tests should be done. Microscopic examination of tissue samples or smears is a commonly used method to diagnose leishmaniasis. Samples are typically taken from skin lesions, bone marrow or lymph nodes. Samples are stained and examined under a microscope to detect the presence of *Leishmania* parasites. With the culture method, it can help to confirm the diagnosis by culturing the parasites from clinical samples. This method involves growing the parasites in a special culture medium such as Dulbecco's Modified Eagle Medium (DMEM) or Roswell Park Memorial Institute (RPMI). The third method, Polymerase Chain Reaction (PCR), is a sensitive technique used to detect and identify the DNA of *Leishmania* parasites. Skin biopsies can be performed on a variety of clinical specimens, including blood, bone marrow, and lymph node aspirates. Serological tests may also detect the presence of antibodies against *Leishmania* parasites in the patient's blood. Common serological tests include the Direct Agglutination Test (DAT), the Enzyme-Linked Immunosorbent Test (ELISA), and the Immunofluorescent Antibody Test (IFAT). Serological tests are helpful in diagnosing visceral leishmaniasis (VL) but may not be as reliable for cutaneous or mucocutaneous forms. As a final laboratory technique, rapid diagnostic tests (RDTs) are immunochromatographic tests that deliver rapid

results within minutes. They detect specific antigens or antibodies associated with *Leishmania* infections. RDTs are simple to use and can be performed at the point of care (12). Finally, imaging techniques can be complementary for the diagnosis of leishmaniasis as well. Ultrasound, X-rays, or computed tomography scans may be used to evaluate specific organs affected by leishmaniasis, such as the liver, spleen, or lymph nodes (12).

Diagnosis method should be chosen by considering the leishmaniasis type (cutaneous, mucocutaneous, or visceral) and the resources available in the healthcare setting. A combination of different diagnostic approaches may be employed to achieve accurate diagnosis and guide appropriate treatment. In addition, consultation with a healthcare professional experienced in diagnosing leishmaniasis is highly recommended.

2.1.5. Treatment of leishmaniasis

The selection of the treatment method partly depends on the host and parasitic factors, and certain approaches may be effective only against specific *Leishmania* species in particular geographic regions. For example, treatment data obtained from various clinical trials for visceral leishmaniasis in certain regions of India may not be applicable to the clinical presentations of leishmaniasis in other regions due to the emergence of drug-resistant *Leishmania* isolates in India.

The dose range and duration of treatment may vary depending on factors, such as the patient's age, weight, immune status, severity of the disease, and the specific *Leishmania* species involved. The dosing regimen should be determined by a healthcare professional experienced in treating leishmaniasis, considering the specific clinical scenario and available treatment guidelines in the region.

All cases of clinically manifest visceral leishmaniasis and mucosal leishmaniasis should be treated. On the other hand, not all cases of cutaneous leishmaniasis require treatment, as some cases may resolve on their own without medical intervention. In

addition, small CL lesions could be treated with cryotherapy as well. There are several drugs in the market today for the treatment of leishmaniasis (Table 1).

Table 1. Drugs used according to clinical classification for leishmaniasis (13).

CL*			**MCL***	**VL***	
Localized Lesions		**Diffuse or Multiple Lesions**	**Systemic**	**First-line treatments**	**Second-line treatments**
Topical treatments	Intralesional injections	Pentavalent antimonials	Pentavalent antimonials	Pentavalent antimonials **	Amphotericin B deoxycholate
Paromomycin ointment	Pentavalent antimonials	Amphotericin B	Amphotericin B	Liposomal Amphotericin B	Amphotericin B lipid
Imiquimod cream	Pentamidine	Miltefosine	Miltefosine	Miltefosine	Paromomycin
					Pentamidine

* Cutaneous Leishmaniasis (CL), Mucocutaneous Leishmaniasis (MCL), and Visceral Leishmaniasis (VL)
** Pentavalent antimonials (Sodium stibogluconate, Meglumine antimoniate)

The drugs used in leishmaniasis treatment can have various side effects (Table 2). Commonly used medications such as the pentavalent antimonials, amphotericin B, miltefosine, paromomycin, and pentamidine may rarely cause adverse reactions as well. It is important for healthcare professionals to monitor patients for these side effects and provide appropriate guidance and management during the treatment of leishmaniasis.

Table 2. Some potential side effects associated with commonly used drugs for the treatment of leishmaniasis (14).

Drug	**Pentavalent antimonials**	**Amphotericin B**	**Miltefosine**	**Paromomycin**	**Pentamidine**
Side Effects	Cardiac toxicity	Infusion-related reactions (fever, chills, hypotension)	Gastrointestinal symptoms (nausea, vomiting, diarrhea)	Nephrotoxicity	Hypoglycemia
	Pancreatitis	Nephrotoxicity	Hepatotoxicity	Ototoxicity	Pancreatitis
	Liver toxicity	Electrolyte imbalances (hypokalemia, hypomagnesemia)	Teratogenicity (should not be used during pregnancy)		Nephrotoxicity
	Nephrotoxicity	Anemia			Cardiac arrhythmias

2.1.5.1. Pentavalent antimonials: meglumine antimoniate

Antimony compounds have been used therapeutically for centuries, with their most important application in the treatment of leishmaniasis (15). In the early 20th century, Gasper Vianna, a pioneering researcher in leishmaniasis treatment, reported the effectiveness of antimony(III) potassium tartrate in the treatment of mucocutaneous leishmaniasis (16). This activity has also been confirmed for visceral leishmaniasis, which is prevalent in places like Italy, India, and Africa. However, due to severe side effects, the clinical use of this compound has been interrupted. Subsequently, less toxic pentavalent antimony (Sb(V)) complexes were included in therapeutic treatments after the 1940s. Although they remain first-line drugs against all forms of leishmaniasis, their use in the clinical setting is limited due to various restrictions. The side effects of the drug are shown in Table 2. The emergence of drug resistance is another significant problem in the treatment of this disease. Second-line drugs such as pentamidine and amphotericin B also exhibit serious side effects, leading to treatment failures (15). In the 21st century, two new drugs were released in the market: a liposomal formulation of amphotericin B (AmBisome®, NeXstar Pharmaceuticals) (17) and miltefosine, which was developed as an anticancer drug and is also used in the treatment of leishmaniasis (Impavido®, Zentaris) (18). While both have shown a cure rate of over 90% in clinical trials for visceral leishmaniasis, the high cost of AmBisome® poses a problem for its use in developing countries, and miltefosine exhibited teratogenicity in animal studies despite its narrow therapeutic window (18). Therefore, the World Health Organization strongly recommends and supports the research for new drugs against leishmaniasis (19).

There are two main antimony compounds used today: N-methyl-D-glucamine antimoniate (meglumine antimoniate or Glucantime®) and sodium gluconate (sodium stibogluconate or Pentostam®). The exact structures of these complexes have remained unknown for decades due to their amorphous state, but significant progress has been made in this field with the use of mass spectrometry and nuclear magnetic resonance (NMR) techniques. Figure 5 shows the approved structures of the dominant

Sb-ligand complex in meglumine antimoniate and sodium stibogluconate solutions (20).

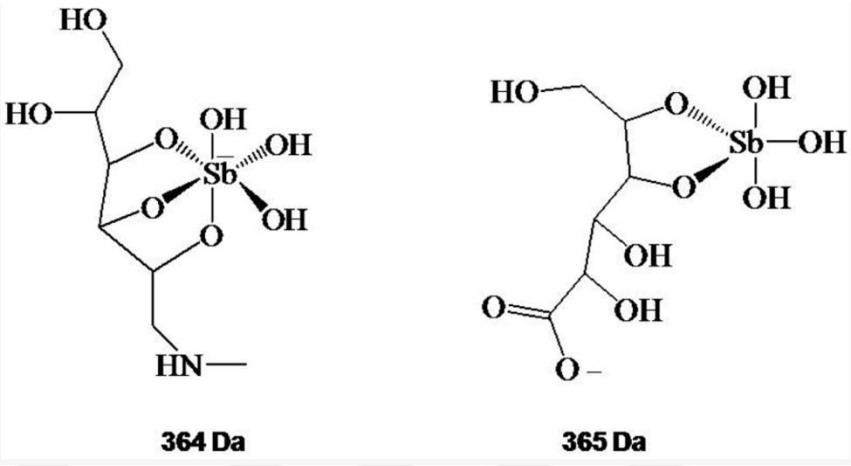

Figure 5. The structural formulas of meglumine antimoniate and sodium stibogluconate in aqueous solutions were determined using ESI(-)-MS and are depicted (20).

The mode of action of pentavalent antimonials against leishmaniasis is still not fully understood. It is unclear whether the ultimate active form of pentavalent antimonials is Sb(V) or Sb(III). Two main models have been proposed, as shown in Figure 6 (15). This model is supported by the observation that a portion of Sb(V) is reduced in vivo to the more toxic Sb(III) (21). It has been shown that mono- and di-thiols, such as glutathione (GSH), cysteine (Cys), cysteinyl-glycine (Cys-Gly), and trypanothione ($T(SH)_2$), act as reducing agents in the reduction of Sb(V) to Sb(III) (22). Thiol-dependent reductases, such as thioredoxin reductase (TDR1) and antimoniate reductase (ACR2), are also reported to contribute to the reduction process (23).

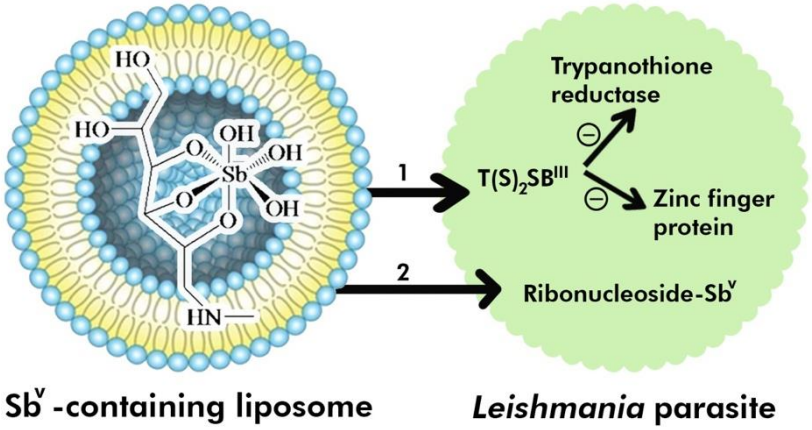

Figure 6. There are two proposed mechanisms through which pentavalent antimonials can affect parasites. In Model 1, they can convert from Sb(V) form into the more toxic Sb(III) form, which can act on trypanothione reductase and zinc finger proteins. In Model 2, pentavalent antimonials can remain as Sb(V) and form complexes with ribonucleosides. As shown in the diagram created using Biorender.com and adapted from Ref (15).

The anti-leishmanial mechanisms of Sb(III) are likely to involve interactions with biomolecules containing sulfhydryl groups, including thiols, peptides, proteins, and enzymes. Sb(III) reacts with $T(SH)_2$ to form $T(S)_2Sb$ complexes. Fairlamb and colleagues recently reported that trivalent antimonials interfere with $T(SH)_2$ metabolism by inhibiting Trypanothione Reductase (TR) (24). In another study, it was reported that Sb(III) binds to a CCHC zinc finger peptide model and supports the release of Zn(II) (25). These structural elements are associated with a wide range of functions, including DNA recognition, RNA packaging, protein folding and assembly, lipid binding, transcriptional activation, cell differentiation and growth, and regulation of apoptosis (26).

According to the second model, Sb(V) exhibits intrinsic anti-leishmanial activity. It has been shown that sodium stibogluconate specifically inhibits type I DNA topoisomerase from *Leishmania donovani*, preventing the relaxation and cleavage of the supercoiled plasmid pBR322, which is not observed with Sb(III), and stabilizing the topoisomerase-DNA covalent complexes (27). Demicheli and colleagues reported the formation of a complex between Sb(V) and adenine ribonucleoside (28). However, the intermediate steps are still under investigation, and only a few hypotheses exist.

These include the inhibition of *Leishmania* purine transporters (29), depletion of ATP and GTP as previously reported after exposure of *Leishmania* parasites to sodium stibogluconate (30), and interference with the purine salvage pathway (29). In conclusion, Sb(V) was shown to be capable of killing the parasites directly and indirectly through various mechanisms.

2.2. *Leishmania* Parasites

Leishmania is an obligatory intracellular protozoan parasite belonging to the *Leishmania* genus. It is a single-celled, eukaryotic organism with a well-defined nucleus and other cellular organelles, such as kinetoplasts and flagella in Figure 7. The kinetoplast is a characteristic feature of these protists, representing highly condensed mitochondrial DNA.

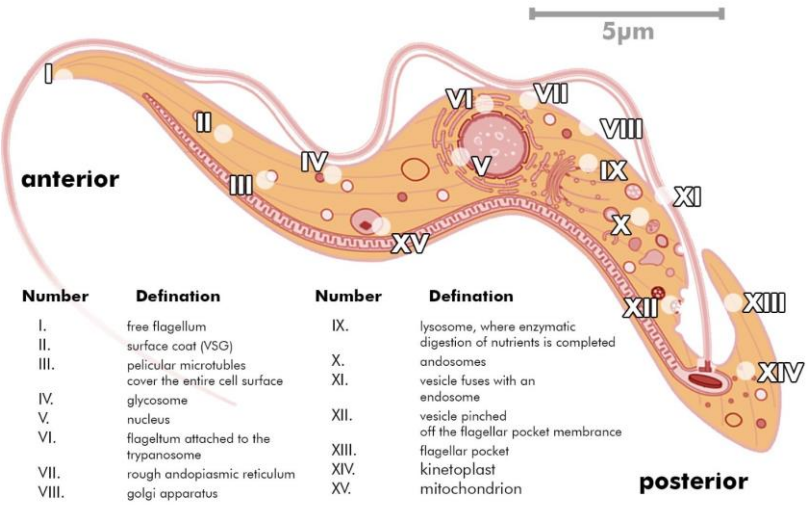

Figure 7. Illustration of *Leishmania* cell in promastigote form. Created in Biorender.com

Mammals and reptiles from the vertebrate class are the main hosts of *Leishmania*. Depending on the primary reservoir, transmission of leishmaniasis can be either zoonotic or anthroponotic (Moreno and Alvar, 2002). For example, in India, *L. donovani* has been reported as the causative agent of anthroponotic visceral leishmaniasis, where humans serve as the main reservoir (Singh, 2006). *Leishmania*

undergoes a transition between two distinct structural forms, namely the amastigote and promastigote forms, to complete its life cycle (Burchmore and Barrett, 2001).

2.2.1. History, discovery, and taxonomy

Fossilized sand flies dating back to the early Cretaceous period have revealed the presence of Paleoleishmania, an ancient genus of the *Leishmania* parasite. The earliest written records describing the prominent symptoms of cutaneous leishmaniasis emerged in the Paleotropics through ancient oriental texts dating back to the 7th century BC (31). Due to its widespread and persistent prevalence as a mysterious disease with various symptomatic outcomes, leishmaniasis has been referred by different names such as "white leprosy" and "black fever (32).

The parasite responsible for the disease was concurrently identified in 1901 by William Boog Leishman and Charles Donovan, who visualized the microscopic single-celled parasites residing in infected human organs. These cells were later named Leishman-Donovan bodies.

Taxonomically, the genus *Leishmania* belongs to the family Trypanosomatidae, order Kinetoplastida, and class Zoomastigophorea. Within the genus, numerous species have been identified, each exhibiting variations in morphology, pathogenicity, and geographical distribution. The classification of *Leishmania* has evolved with advances in molecular techniques, such as DNA sequencing and phylogenetic analysis. Since then, several species have been classified and grouped under two main subgenera, namely *Leishmania* Viannia (mostly found in the Neotropics) or *Leishmania Leishmania* (generally found in the Paleotropics, except for the L. mexicana subgroup) as shown Figure 8 (33).

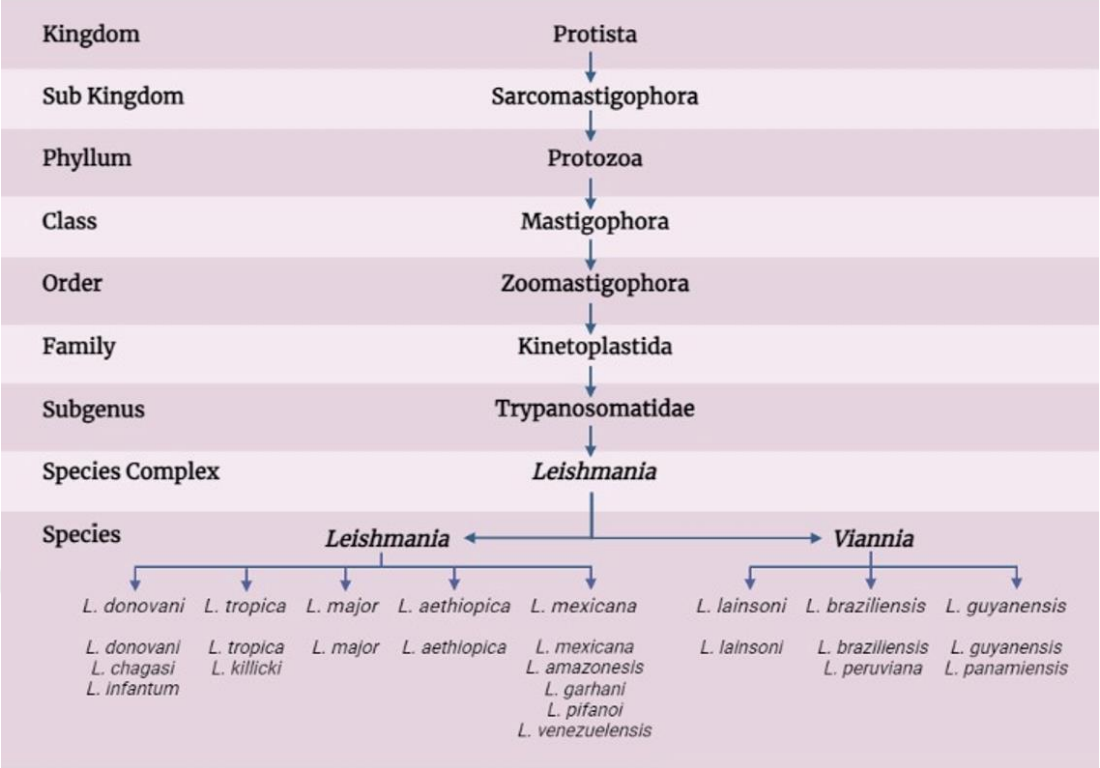

Figure 8. Leishmania genus can be classified into hierarchical taxonomical categories. Within the *Leishmania* species complex, there are five species that are known to cause visceral, mucocutaneous, and cutaneous leishmaniasis. Additionally, within the *Viannia* species complex, there are three species are classified associated with these forms of leishmaniasis. Created in Biorender.com and adapted from Ref (34).

2.2.2. Life cycle

The life cycle of *Leishmania* begins when a female phlebotomine sandfly ingests blood from an infected mammalian host, containing the infective stage of the parasite called promastigotes. These forms are characterized by their elongated needle-like shape, measuring approximately 15-30 μm in length and 5 μm in width, with a long flagellum protruding from the anterior end (Figure 6). The blood meal serves as a source of nutrients for the sandfly's egg production. Inside the sandfly's midgut, promastigotes undergo transformation into procyclic promastigotes and undergo multiplication. Kamhawi et al. (2015) investigated the interactions between *Leishmania* promastigotes and the sandfly vector. They discovered that upon ingestion by the sandfly, promastigotes undergo molecular and physiological changes, including alterations in gene expression, metabolism, and surface molecule composition. The researchers identified specific molecules involved in the attachment of promastigotes

to the sandfly's midgut, enabling the parasites to establish infection and complete their development (35).

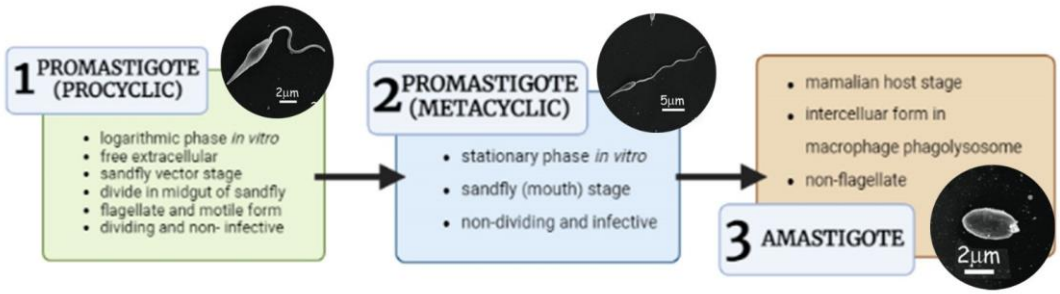

Figure 9. The stages of *Leishmania* are as follows: procyclic stage in the promastigote form, metacyclic stage in the promastigote form, and amastigote form. Created inBiorender.com

As procyclic promastigotes multiply, they differentiate into infective metacyclic promastigotes, which are the form capable of infecting mammalian hosts in Figure8. Metacyclic promastigotes migrate to the proboscis of the sandfly and can be injected into a mammalian host during the next blood meal. In Figure 9 depicted above, *Leishmania* in the procyclic stage reaches the logarithmic phase of the growth curve, while in the stationary phase, it transitions to the metacyclic stage in the promastigote form. It is observed that metacyclic stage is more infective compared to the procyclic stage. Telleria et al. (2015) conducted a study focusing on the molecular events involved in the differentiation of *Leishmania* promastigotes into infective metacyclic forms (36). By highlighting the genes involved in metacyclogenesis to uncover the significant molecular events occurring during this differentiation process, they identified stage-specific gene expression patterns. They also discovered significant changes in surface molecule composition, such as the upregulation of specific surface glycoproteins. These molecular changes facilitate the migration of metacyclic promastigotes to the proboscis of the sandfly and enhance their ability to infect mammalian hosts.

When a sandfly bites a mammalian host, metacyclic promastigotes are injected into the skin. Within the host, they are phagocytized by macrophages and transform

into amastigotes. Amastigotes are oval-shaped, non-flagellated, immotile forms measuring approximately 3-6 μm in length. They are also the form observed during the diagnostic stage of the disease in humans. Amastigotes proliferate within the phagolysosomes of phagocytic cells such as macrophages, neutrophils, and dendritic cells, contributing to the progression of the disease. When an infected sandfly bites another mammalian host, it introduces infective promastigotes, thus completing the transmission cycle. Whole cycles are shown in Figure 10.

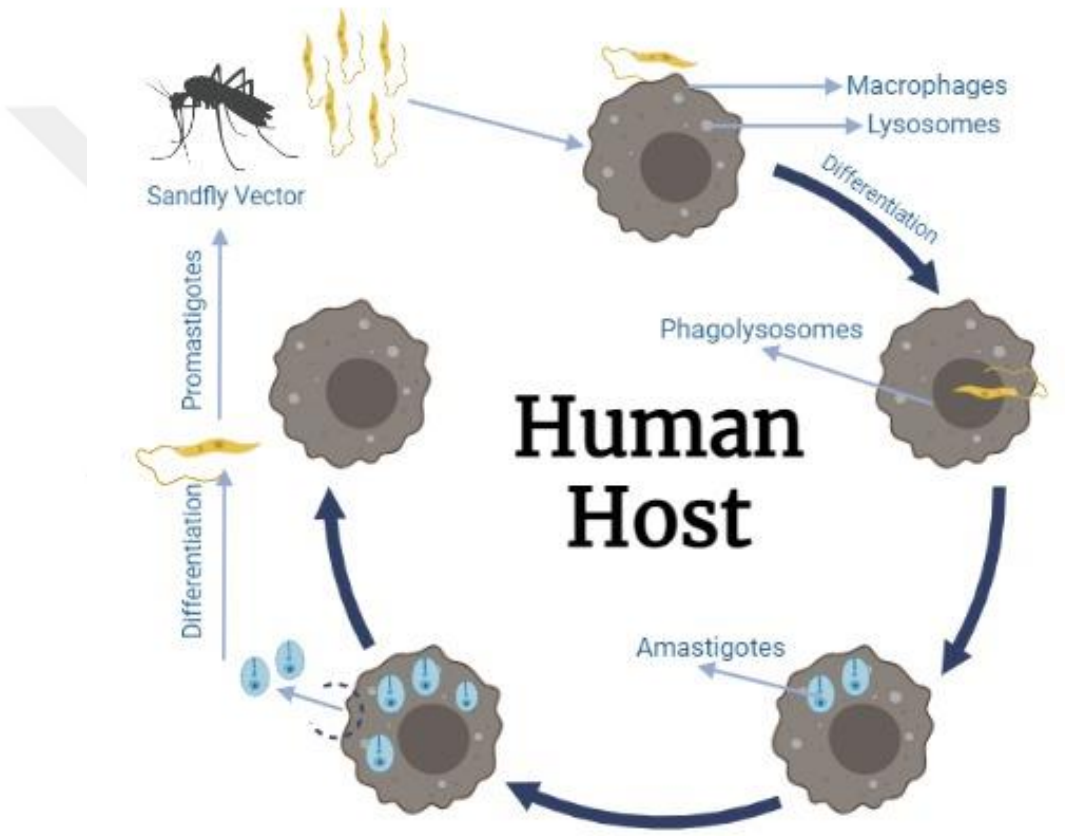

Figure 10. The life cycle of Leishmania. The illustration was created using Biorender, taking inspiration from Raj et al.'s drawing (34).

2.2.3. Pathogenicity

Genetic and molecular characteristics that influence the pathogenicity in *Leishmania* species include the expression of virulence factors, their impact on immune responses, and their ability to adapt to the host.

2.2.3.1. Virulence factors

Virulence factors are the components that influence the ability of pathogenic microorganisms to cause disease and affect the infection process. These factors enable the parasite to survive, proliferate, and spread successfully within the host organism. In *Leishmania* species, virulence factors can have an impact on the severity of the disease, the course of infection, and the effectiveness of treatment strategies. Main virulence factors of *Leishmania* species are presented below:

Lipophosphoglycan (LPG) is a glycolipid compound found in the cell wall of *Leishmania* species and plays a significant role in pathogenicity. In *L. major*, LPG could bind to host cells and modulate immune responses (37).

Glycoprotein 63 (GP63) is a surface glycoprotein found in *Leishmania* species. It facilitates the entry of parasites into the host cells and has a range of pathogenic effects, including binding to host cells, inhibiting phagocytosis, and modulating immune responses (38).

Leishmania species are proficient in producing enzymes called metalloproteases. These enzymes play a role in processes such as parasite entry into host cells, binding to host cells, tissue invasion, and suppression of immune responses (39).

Cysteine peptidases play a crucial role in the parasite's ability to establish infection and evade the host immune response. These proteases contribute to various aspects of *Leishmania* pathogenesis, including tissue invasion, modulation of host cell signaling, and degradation of host immune factors. A study conducted by Dey et al. (2018) focused on *L. major* and demonstrated the involvement of cysteine proteases in parasite survival and immune evasion. They identified specific cysteine protease genes that are upregulated during infection and play essential roles in parasite virulence.

Leishmania species possess kinetoplast DNA, also known as mitochondrial DNA. kDNA plays a vital role in *Leishmania'*s energy metabolism and is necessary for

parasite survival. Additionally, kDNA replication and transcription are factors that affect the parasite's proliferation within host cells and their pathogenicity (40).

2.2.3.2. Impact on the immune responses

Leishmania species have various effects on immune responses, which can determine the course of infection and the severity of the disease. The main areas of impact include functional alterations in macrophages, cytokine imbalances, and the modulation of immune regulatory cells. Inspired by several studies on *Leishmania*, factors involved in immune response modulation have been identified:

Cysteine protease A (CPA) is an enzyme found in *Leishmania* species that plays a crucial role in regulating immune responses. It has been shown that in L. *donovani*, CPA suppresses inflammation induced by the immune system, facilitating the parasite survival (41).

Interferon-gamma (IFN-γ) Secreting Cytokines play an important role in regulating antiparasitic immune responses. *Leishmania* species such as *L. major* activate T cells that secrete IFN-γ from host cells. This immune response is critical for controlling parasites and limiting infection (42).

Toll-Like Receptors (TLRs) play a significant role in activating immune responses during *Leishmania* infection. *Leishmania* species like *L. major* induce cytokine release and inflammation by activating TLRs in host cells. These molecular interactions influence the course of infection and the nature of immune responses (43).

Leishmania species produce various antigens that shape immune responses. For instance, surface antigens like lipophosphoglycan (LPG) play a significant role in regulating immune responses during *Leishmania* infection. LPG affects cytokine release and modulates immune responses by binding to host cells (44).

2.2.3.3. Adaptation ability

2.2.3.3.1. Drug resistance

The mechanisms of drug resistance in *Leishmania* species can develop based on the genetic and molecular characteristics of the parasite. By utilizing carrier proteins called drug pumps located on the cell membrane, some *Leishmania* species can develop resistance to drugs by pumping them out of the cell. These pumps can prevent the entry of drugs into the cell or retain them at ineffective concentrations, reducing the parasite's exposure to the drug (45).

Leishmania can alter their metabolic pathways or produce enzymes that inactivate drugs, thereby changing drug metabolism and developing resistance. For example, some *Leishmania* species can gain resistance to drugs such as miltefosine, a phospholipid-based compound, by inactivating it through phosphorylation mediated by phosphotransferase enzymes (46)

Furthermore, *Leishmania* species can acquire resistance to drugs by developing mutations in the target proteins where the drugs exert their effects. For instance, antimony resistance in *Leishmania* species can be associated with mutations in a parasite's dihydrofolate reductase enzyme. These mutations cause structural or functional changes in the target enzyme, leading to the development of drug resistance (47).

2.2.3.3.2. RNA viruses and pathogenicity

The adaptation ability of RNA viruses to *Leishmania* species is a significant factor that can influence the pathogenicity and infection process of the parasite. RNA viruses can alter the pathogenicity of the parasite, thereby modifying the course of infection. For example, *Leishmania* RNA virus (LRV) can increase the replication rate of infected parasites and alter immune responses. This can affect the severity and clinical progression of the disease. Additionally, RNA viruses can influence the adaptation

capacity of infected parasites by modifying immune responses. By modulating immune responses, they can enable infected parasites to develop immune evasion mechanisms. This can result in better hiding of the parasite becoming from the immune system and development of chronic infection. LRV can also interact with the parasites, leading to genetic material exchange. These interactions can increase the genetic diversity of the parasite and enhance its adaptation ability. It is also suggested that LRV can induce permanent changes in the parasite's genome (48).

A study conducted by Zangger et al. (2014) examined the impact of *Leishmania* RNA virus-1 (LRV-1) in *L. guyanensis* on the pathogenicity of the parasite. They found that LRV-1 increased the replication rate of infected parasites and triggered the production of proinflammatory cytokines, indicating that LRV-1 can enhance the parasite's adaptation ability and influence the course of the infection (49). Rahmanipur et al. (2023) investigated the pathogenicity of Leishmania RNA virus-2 (LRV-2) found in L. major on THP-1, a human leukemia monocyte cell line. It was observed that LRV-2 enhances the survival probability of the parasite within the cells (50).

2.2.4. Metabolic pathways of *Leishmania*

The growth and replication of *Leishmania* parasites are influenced by various metabolic pathways, such as sterol biosynthesis, purine salvage, glycosylphosphatidylinositol (GPI) biosynthesis, folate biosynthesis, hypusine, and trypanothione pathways (51). Therefore, these pathways become essential drug targets against *Leishmania* infection. In the process of drug development, the first step involves selecting a target within the parasite's biochemical pathway that is either absent or significantly different from the host counterpart (52). Additionally, the chosen target should be involved in a vital metabolic pathway or cellular function that can effectively eliminate or inhibit the growth of the parasite (53)**.** The main metabolic pathways for *Leishmania* species are illustrated in Figure 11.

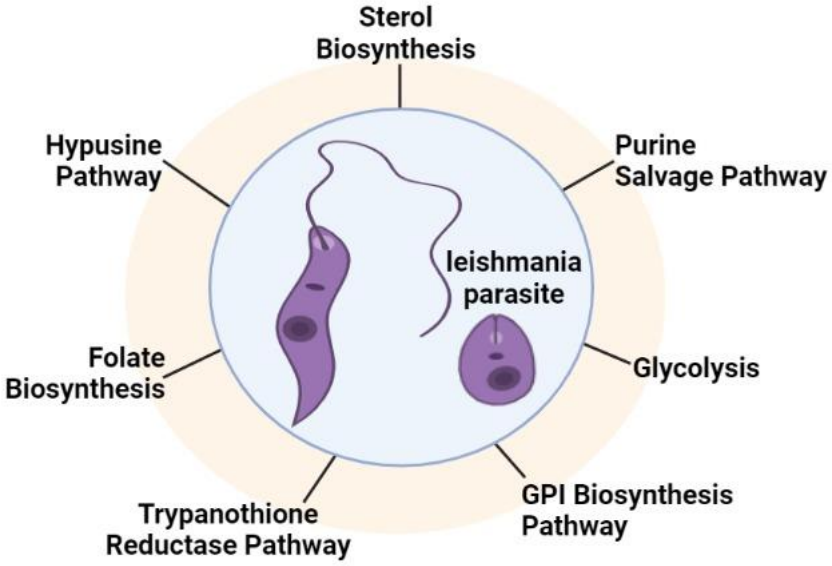

Figure 11. The most critical metabolic pathways in Leishmania species. Created in Biorender. Adapted from Ref. (54)

In human pathogenic stages of various trypanosomatid parasites like *Leishmania*, sterol production has been reported as a crucial metabolic process. Sterols, which serve as precursors and regulators in cell cycle progression and development, are key components regulating membrane fluidity and permeability in eukaryotic cells (55). The main sterol lanosterol is converted to cholesterol in mammals, while in trypanosomatids and fungi, it is converted to ergosterol. Therefore, in *Leishmania*, the sterol biosynthetic pathway produces ergosterol, which replaces cholesterol found in the host (56). Additionally, traditional anti-leishmanial drugs such as Amphotericin B, miltefosine, and azoles disrupt the permeability barrier of the cell membrane by inhibiting or disrupting the sterol biosynthesis pathway (56).

Another pathway that differs from the host is the purine salvage pathway. While mammalian cells can synthesize purine nucleotides from amino acids and one-carbon compounds, protozoan parasites cannot produce them de novo. One effective technique for targeting the purine salvage pathway is the Purine Pyrimidine Analog (PPA) technique, which focuses on creating prodrugs of purine ribonucleosides that are inactive in mammals but activated in parasites, leading to the production of harmful metabolites (57).

Glycosyl Phosphatidyl Inositol (GPI) Biosynthesis involves the sequential addition of sugar and ethanolamine to phosphatidylinositol (PI) (58). GPI molecules assist in the survival and proliferation of *Leishmania* parasites in both hosts and act as anchors for several important surface molecules, including Lipophosphoglycan (LPG), glycol inositol phospholipids (GIPLs), glycoproteins (GP63), and proteophosphoglycan (PPG). For example, the interaction of GPI-14 with anchored chemicals revealed inhibition of mannosylation, suggesting the potential application of drugs for leishmaniasis treatment (59).

In the Folate Biosynthesis pathway, tetrahydrofolate functions are essential for the biosynthesis of purines, thymidylate, pantothenate, RNA, and amino acids (60). While eukaryotes can acquire folate from the environment, certain steps of the folate pathway, such as dihydrofolate reduction and glutamate tetrahydrofolate formation, are conserved (61). However, it is necessary to focus on metabolic pathways associated with the transfer of one-carbon units in Leishmania parasites, which differs from eukaryotes (62). For example, Dihydrofolate Reductase (DHFR) is an important enzyme in folate metabolism and thymidine synthesis. Methotrexate (MTX, 1), cycloguanil, and trimethoprim (TMP, 2) are DHFR inhibitors used therapeutically, containing various bioactive moieties (63).

The parasite's survival in the host's immune response against infection is linked to the oxidative stress intensity associated with the trypanothione pathway. In infectious trypanosomatids, Trypanothione (TSH2), the major redox reactive metabolite, participates in various activities by transferring electrons to numerous acceptors involved in survival mechanisms such as peroxide detoxification and DNA synthesis (64) (65). As trypanosomatids lack redox mechanisms, they rely on TSH2, which plays a crucial role in thiol homeostasis (64) (66). The absence of this pathway in humans presents a unique opportunity for the discovery of drugs that can prevent the parasite's survival without harming the host (67).

2.2.5. *Leishmania major*

Leishmania major is a parasitic species belonging to the *Leishmania* genus, which causes an infection known as tegumentary leishmaniasis (cutaneous leishmaniasis) in humans. It was first identified by William Boog Leishman in 1903 in military personnel in Sudan, and he named the disease as "Kala-Azar." Subsequently, the presence of different species within the *Leishmania* genus was discovered, and it became evident that these species cause different clinical forms of the disease.

Leishmania major primarily causes infection inside the skin tissues, while other species can also cause infections in internal organs. Moreover, there are genetic and molecular variations among different *Leishmania* species, which can influence treatment strategies. The first-line treatment for *Leishmania major* infection usually involves antimonial compounds (such as sodium stibogluconate) or oral antiparasitic drugs like miltefosine. These medications work by stopping the growth and proliferation of the parasite, thus controlling the infection. Other treatment options may include drugs like amphotericin B, paromomycin, and pentamidine. The genomic, molecular expression, immune response differences, and drug resistance observed in *L. major* distinguish it from other *Leishmania* species.

2.2.6. Leishmania RNA virus

Leishmania RNA virus (LRV) is a double-stranded RNA virus in the family of Totiviridae. It serves as a natural host for protozoa and is found in various species of the protozoan parasite *Leishmania* (68). The presence of virus-like particles in *Leishmania* species was first reported in *Leishmania hertigi* in 1974.

There are two subtypes of LRV; LRV1 is found in New World *Leishmania* strains such as *L. guyanensis* and *L. braziliensis,* while LRV2 is found in the Old World *Leishmania* strains (*L. major, L. aethiopica, L. tropica, and L. infantum).* The virus has an approximate size of 40 nm and encodes capsid protein and RNA-dependent RNA polymerase (RDRP) within a 4.8 kb double-stranded RNA (dsRNA) (69).

LRV (*Leishmania RNA virus*) can lead to a destructive hyperinflammatory response that is associated with the severity of infection and parasitic metastasis. Previous studies conducted on human cases have indicated that LRV can worsen the clinical prognosis of *Leishmania* spp. cutaneous leishmaniasis (CL) and potentially contribute to the development of mucocutaneous leishmaniasis (MCL), which may result in treatment failure (70).

2.3. Fungi as a Source of Biomedical Compounds

Fungi have been acknowledged for their significant contribution as valuable reservoirs of diverse bioactive compounds with potential biomedical applications. These compounds, encompassing secondary metabolites and enzymes, demonstrate a wide range of pharmacological activities, such as antimicrobial, antifungal, antiparasitic, anticancer, and immunomodulatory properties.

2.3.1. Fungi as a source of antileishmanial compounds

Bioactive compounds derived from fungi offer a promising avenue for the development of new drugs against leishmaniasis. Fungi produce a wide range of secondary metabolites with diverse chemical structures and biological activities. Many of these metabolites have demonstrated significant antileishmanial potential. For instance, compounds derived from fungal sources such as terpenoids, alkaloids, polyketides, and peptides have shown antileishmanial activity in various studies.

These compounds exert their effects on *Leishmania* parasites through different mechanisms. These mechanisms may involve disrupting the parasite's membrane integrity, inhibiting key enzymes or metabolic pathways, modulating host immune responses, or inducing apoptosis in parasite cells. Ergosterol, for example, is a vital component of the cell membranes of protozoa and fungi. Azole drugs like ketoconazole and allylamine drug terbinafine, which can inhibit ergosterol biosynthesis, exhibit efficacy against leishmaniasis (71).

Amphotericin B, a polyene antifungal drug, functions by binding to ergosterol in fungal cell membranes, forming pores that disrupt membrane integrity and cause cell death. Interestingly, several species of *Leishmania* parasites also possess ergosterol-like molecules, such as ergosta-5,7,22-trienol, in their cell membranes. This structural similarity enables amphotericin B to interact with and affect the membranes of *Leishmania* parasites, exerting its antileishmanial activity (72).

Furthermore, fungal peptides such as antifungal defensins and cyclic peptides have also shown potential as antileishmanial agents. These peptides can disrupt the integrity of parasite membranes or interfere with vital cellular processes.

2.3.2. Effective fungal peptides on leishmaniasis

Fungal peptides, also known as antimicrobial peptides (AMPs), are naturally occurring molecules produced by fungi as part of their defense mechanisms against pathogens. Most AMPs are cationic and amphipathic in nature. These peptides are typically short, ranging from 10 to 50 amino acids in length, and display a wide range of structural and functional diversity. They unite under two main functions: direct killing of pathogens and modulation of the immune response (73). While the antibacterial effects of AMPs are well-known, they also exhibit antifungal, antiviral, and antiparasitic activity. The presence of AMP families with anti-leishmanial effects, which share similar mechanisms with drugs used to treat leishmaniasis, is known as shown in Figure 12.

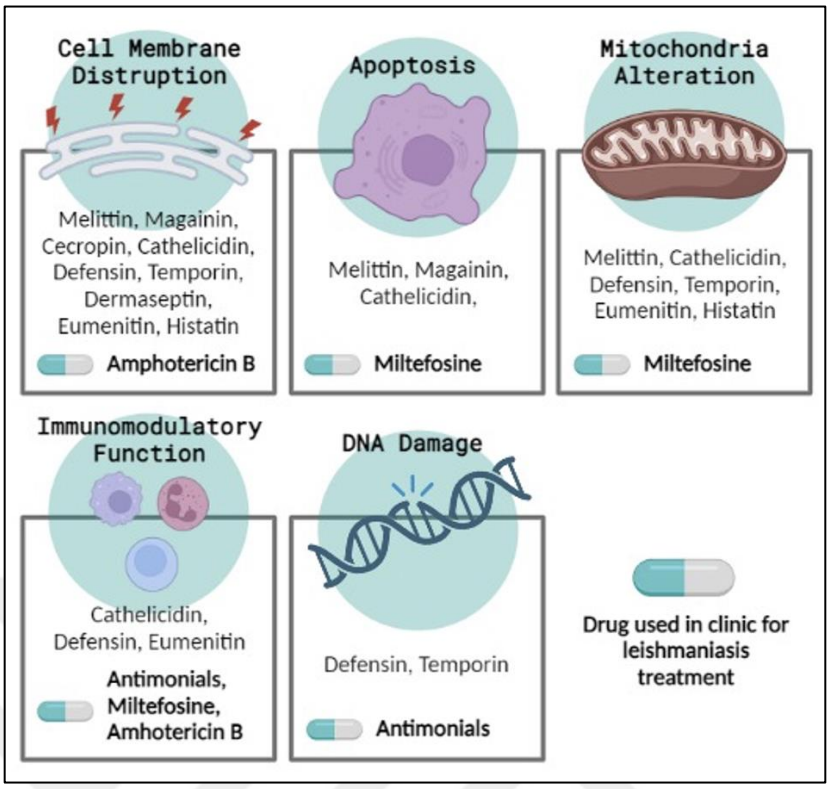

Figure 12. The similarity in mechanisms of action between different antimicrobial peptide (AMP) families and the drugs used to treat leishmaniasis**.** Created in Biorender.com and adapted from Ref (73).

For instance, defensins, a family of AMPs, play a crucial role in the host defense against microbial infections by inducing innate immune cells such as mast cells, T lymphocytes, dendritic cells, and others. Recently, an experiment was conducted using a C57BL/6 mouse model, known to be resistant to *Leishmania* infections, to investigate the potential underlying mechanisms of resistance. It was found that the resistance to *Leishmania* species may be attributed to the involvement of additional immune systems. *L. major* parasites induce the secretion of interleukin (IL)-12 in macrophages, while in this mouse model, T lymphocytes enhance immunity by inducing the expression of interferon-gamma (INF-γ) cytokine (74).

2.3.3. Potential of fungi for developing new target agents in *Leishmania* treatment

Several studies have investigated the antileishmanial activity of fungal peptides, highlighting their potential as therapeutic agents against leishmaniasis. In a study

conducted by Dutta et al. (2019), the leishmanial activity of a fungal peptide called Coprisin, derived from the dung beetle *Copris tripartitus*, was evaluated. The researchers found that Coprisin exhibited significant antileishmanial activity against both promastigotes and amastigotes of *Leishmania donovani*, the causative agent of visceral leishmaniasis (75). Furthermore, it was discovered that an endophytic fungus, *Cochliobolus sp.*, effectively killed 90% of *Leishmania amazonensis*' amastigote form. The chromatographic fraction of the extract revealed the presence of active compounds, kokliokinone A and isokokliokinone A (76). Overall, these findings demonstrate the potential of fungal peptides such as Coprisin and natural products from endophytic fungi like Cochliobolus sp. as promising candidates for the development of novel antileishmanial therapies.

2.3.4. *Aspergillus* and their hydrophobin structures

Hydrophobins are low molecular weight (approximately <20 kDa) and cysteine (Cys)-rich amphipathic proteins commonly found in filamentous fungi. Despite having low amino acid similarity, hydrophobins share specific characteristics such as the presence of eight conserved Cys residues and four disulfide bonds. They also exhibit similar β-barrel structures. While some filamentous fungi, such as *Aspergillus*, *Penicillium*, and *Trichoderma*, possess numerous hydrophobin-coding genes, many other filamentous fungi have only a few. The expression levels of hydrophobin genes necessary for their production are dependent on culture conditions (77).

Hydrophobins are secreted by filamentous fungi and self-assemble on liquid or air surfaces. This phenomenon reduces interfacial surface tension, facilitating the formation of aerial hyphae and conidia. Hydrophobins particularly accumulate within aerial hyphae and exhibit the highest production during sporulation. The hydrophobic nature of the filamentous fungi surface aids in the absorption of these fungal species onto host insects and plants. Hydrophobins deposited on solid surfaces can bind various proteins, such as bovine serum albumin, chitinase, and IgG (78).

The *Aspergillus* genus belongs to the ascomycetes and is polyphyletic (79). *Aspergillus* species are widely used in the fermentation industry and are highly effective in the degradation of solid polymers. Due to their ability to produce primary and secondary metabolites with high efficiency, Aspergilli are employed as host microorganisms in industries (80). Specifically, *Aspergillus oryzae* and *Aspergillus niger* are extensively utilized for the production of enzymes such as amylases, cellulases, and glucosidases (81). The biological effects of *Aspergillus* hydrophobins have been studied for the past 30 years.

Hydrophobins are divided into two classes: Class I and Class II. Class I hydrophobins include those from *Aspergillus* species such as RodA from *Aspergillus fumigatus* (AfRodA), DewA from *A. nidulans*, and RolA (HypA) from *A. oryzae* (82). Class I hydrophobins are further classified into two groups: IA ascomycetes and IB basidiomycetes. Class I hydrophobins form self-assembled structures called rodlets, which consist of β-amyloid fibrils (83). Rodlets are soluble in trifluoroacetic acid (TFA) but have very low solubility in HCl, NaOH, SDS, or ethanol. They are observed in structures such as hyphae and conidia. Rodlets rich in cysteine residues can form loops between Cys3–Cys4, Cys4–Cys5, and Cys7–Cys8 (84).

In Class II hydrophobins, several filamentous fungi such as Trichoderma spp., Fusarium spp., and Neurospora spp. are included, but *Aspergillus* is not classified in this group. Instead of rodlet structures, Class II hydrophobins exhibit self-assembling single-layered films. They are soluble in HCl, NaOH, SDS, ethanol, and TFA (85)

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. *Leishmania* strains

The strains used in this thesis are *Leishmania major* isolates obtained from two patients, one with LRV+ (*Leishmania* RNA virus-positive) and the other without LRV (LRV - [*Leishmania* RNA virus-negative]) strains. The LRV+ *Leishmania* major strain was isolated from a school girl in Manisa, located in Western Anatolia, 16 years ago. The girl presented with a 6 month old, 4 cm ulcer on her left ear, due to leishmaniasis. Initially, intralesional treatment with 20 mg/kg Glucantime® (meglumine antimoniate) was administered three times a week for three weeks, but no improvement was observed. Consequently, the treatment protocol had to be repeated twice. These two strains were obtained from Prof. Dr. Ahmet Özbilgin, from the Parasitology Department at Celal Bayar University in Manisa, Turkey. These isolates have been stored at -80 ˚C in the Acibadem University Research Laboratory.

3.1.2. *Leishmania* culture

Cultivation of Leishmania isolates were made using RPMI medium. The medium ingredients for *Leishmania* strains are as listed in Table 3.

Table 3. The solutions were utilized for *Leishmania* cell culture experiments.

Medium/Reagents	**Supplier Company**
Roswell Park Memorial Institute (RPMI) Medium 1640 with L- Glutamine	GIBCO®
Fetal Bovine Serum (FBS)	GIBCO®
Penicilin/Streptomycin	GeneMark® (Taiwan)
Gentamicin	GIBCO®

3.1.3. Fungi strains

Strains of *Aspergillus* species encoded as 2874, 3508, 3616, and 3766, which were previously obtained from TU Collection of Industrial Microorganisms (TUCIM) located in Vienna, Austria, were used in this thesis. These strains were collected from the tropical rainforests of Borneo, the largest island in Asia, known for its diverse flora. The strains have been stored in the research laboratory of Acibadem University at -80°C. Additionally, a strain labeled as Uşak6, which is suspected to be a species closely related to *Aspergillus* based on fungal samples collected from Uşak province in Turkey by Assoc. Prof. Dr. Günseli Bayram Akçapınar, was utilized. The Uşak6 strain is stored under the same conditions as the TUCIM samples.

3.1.4. Fungi culture

The *Aspergillus* strains used in this thesis were cultured in Malt Extract Agar, Malt Extract Broth, and *Aspergillus* Minimal Liquid Medium (AMM). The recipe for *Aspergillus* Minimal Medium was obtained from a study conducted by Scott et al. (2022) (35). The necessary materials are listed in this section, while the quantities of chemicals are described in the methods section. The equipment used is presented under the "3.1.6 Equipments" heading, and Table 4 provides information about the chemicals, ingredients, and media used.

Table 4. The ingredients used in the Malt Extract and *Aspergillus* Minimal Medium.

Medium/ Chemicals	**Supplier Company**
Agar	Sigma-Aldrich
Ammonium Molybdate Tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$)	Sigma-Aldrich
Boric Acid (H_3BO_3)	Wisent Bioproducts
Cobalt (II) Chloride ($CoCl_2$. H_2O)	Sigma-Aldrich
Copper Sulfate Pentahydrate ($CuSO_4.5H_2O$)	Sigma-Aldrich
Dextrose (D-glucose)	Wisent Bioproducts
Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA)	Sigma-Aldrich
Iron (II) Sulfate Heptahydrate ($FeSO_4.7H_2O$)	Sigma-Aldrich
Magnesium Sulfate Heptahydrate ($MgSO_4.7H_2O$)	Sigma-Aldrich
Malt Extract Broth	Merck
Manganese (II) Chloride ($MnCl_2.4H_2O$)	Sigma-Aldrich

Table 4. (Cont'd) The ingredients used in the Malt Extract and *Aspergillus* Minimal Medium.

Medium/ Chemicals	**Supplier Company**
Potassium Chloride (KCl)	Sigma-Aldrich
Potassium Hydroxide (KOH)	Merck
Potassium Phosphate Dibasic (KH_2PO_4)	Sigma-Aldrich
Sodium Hydroxide (NaOH)	Sigma-Aldrich

3.1.5. Primers

Prior to the completion of the primer designs, a preliminary work was conducted which is described briefly as follows: If the *Leishmania* gene had been previously identified and a FASTA sequence was generated, the sequence of the relevant region was obtained from the National Library of Medicine (NIH). If only the protein corresponding to the gene was available, the protein sequence was obtained from the UniProt.org website, and BLAST was performed using the protein-translated-nucleotide tool at the National Center for Biotechnology Information (NCBI). The region to be amplified was selected, and primers were designed using the Primer3Plus program based on the regions in its vicinity. Subsequently, the primers were verified using the NCBI Primer-BLAST tool and then they were synthesized. The primers used in the study are shown in Table 5.

Table 5. Primer was used to find gene expression levels of *Leishmania* strains.

Primer Name	**Gene Name**	**Forward Primer Sequence**	**Reverse Primer Sequence**	**Product Size**
DHFR	Dihydrofolate Reductase-thymidylate Synthase	**5'**-CAGGATGTGGTGGTGGTGAA-**3'**	**5'**-CGTAGATGCGGGTCAGGTAC-**3'**	178 bp
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase	**5'**-TGAAGACGCCAGATGTGCTT-**3'**	**5'**-GGCCAGTAGACTCGATCACG-**3'**	123 bp
GP63	Leishmanolysin	**5'**-TTCCAGGACCTCGGCTTCTA-**3'**	**5'**-TTCCAGGACCTCGGCTTCTA-**3'**	141 bp
GPI	Mannosyltransferase	**5'**-CGACGAGGGCTTCATCTACC-**3'**	**5'**-CAGAAACGCAAACAGTCCGG-**3'**	142 bp
KMP-11	Kinetoplastid Membrane Protein 11	**5'**-AGATGCAGGAACAGAACGCC-**3'**	**5'**-TGCTTGAAGTGCTCCGAGTG-**3'**	160 bp
LRV2	*Leishmania* RNA Virus 2	**5'**-AGACTGCAACGTGTGGACAT-**3'**	**5'**-ACCGTCATTGTCTTCCGTCC-**3'**	155 bp
TRYR	Trypanothione Reductase	**5'**-GGCTGCGTCCCTAAGAAACT-**3'**	**5'**-TTGATGCCGTTCACCACCTT-**3'**	155 bp

3.1.6. Equipments

The laboratory equipment used throughout the study have been presented in Table 6.

Table 6. List of equipment used throughout the study.

Equipments	**Equipments**
1.0 mm Zirconia beads	Biospec Products®
96-Well Flat Bottom Plate	Sarstedt®
Analytical Balances	Kern Uni Bloc®
Autoclave	Nüve® 90L
Beatbeater	Scientific Industries®, Vortex Genie 2
Biosafety Class II Cabinet	Thermo Scientific® Safe 2020®
Centrifuges	Thermo® SL16, MicroCL 21R
Cryotubes	Isolab®
Dry Heat Blocks	Witeg®, FinePCR
Dry Heat Sterilizer	Nüve® FN 120, Heratherm Oven
Electronic Pipette Filler	Accupetta®
Electrophoresis System	Major Science ®
Falcon Tubes	Isolab®, Labmarker®
Fluorescence Microscope	ZEISS ®AXIO Vert.A1
Fridge	Arctiko®, Kirsch®
Imaging System	Bio-rad® ChemiDoc
Incubator	Thermo Scientific® Heratherm
Light Microscope	Leica® DM500
Magnetic Shaker with Heat	Isolab®
Microplate Spectrophotometer	Thermo Scientific® Varioskan Flash, Biotek Powerwave XS2
Microwave	Arçelik
Nanodrop	Thermo Scientific® Nanodrop One^{C}
Ph Meter	VioLab®
Pipette Tips	Ratiolab, ® Isolab®
Pipettes	Thermo® Finnpipette
Power Supplier	Bio-Rad®
Real Time PCR	Bio-Rad® CFX-96^{TM} C1000 Touch
Serological Pipette Tips	Isolab, ® Falcon®
Shaking Incubator	BioSan®
T25 Cell Culture Flask	TPP®
Thermal Cycler, PCR	Bio-Rad® T100
Ultrasonic Water Bath	Bandelin Sonorex®
Vortex	BioSan® FV2400N
Water Bath	Waterlab Instruments®
Water Purification System	Merck® Milli-Q® Advantage A10

3.1.7. General chemicals and commercial kit

The general chemicals and commercial kit used throughout the study have been presented in the Table 7 and Table 8.

Table 7. List of chemicals used during all study.

Chemicals	**Supplier Company**
6X DNA Loading Dye	GeneMark ®
50bp DNA Ladder	Invitrogen®
50X TAE Buffer	Bioland®
ß-mercaptoethanol	Sigma-Aldrich®
Agarose	Sigma-Aldrich®
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich®
Ethanol 100%	Merck ®
Ethylenediaminetetraacetic acid	Merck®
Glycerol	Research Product International®
Hydrochloric Acid (HCl)	Merck®
PBS 10X	GeneMark®
Potassium Chloride (KCl)	Sigma-Aldrich®
Potassium Hydroxide (KOH)	Merck®
Potassium Phosphate Monobasic (KH_2PO_4)	Sigma-Aldrich®
Sodium Chloride (NaCl)	Sigma-Aldrich®
Sodium Hydroxide (NaOH)	Merck®
Sodium Phosphate Dibasic (Na_2HPO_4)	Sigma-Aldrich®
Syber Safe 20000X	Ecotech®
Trifluoroacetic Acid (TFA)	Merck ®
Tris Base	Merck®
Tween80 (Polyoxyethylenesorbitan)	Research Product International®

Table 8. List of commercial kit used during all study.

Commercial Kit	**Catalog number**	**Supplier Company**
480 SYBR Green I Master	04 707 516 001	LightCycler®
High-Capacity cDNA Reverse Transcription Kit	4368814	Applied Biosystems®
Metabolism Assay Kit	E-BC-K318-M	Elabscience®
PCR Master Mix II (5X)	RP02-II-2000	GeneMark®
Tissue&Cell Genomic DNA Purification Kit	DP021	GeneMark®
Total RNA Purification Kit	TR01	Bioelegen ®
XTT Cell Viability Kit	30007	Biotium®

3.1.8. Other statistics, analysis, and graphics tools

GraphPad Prism 9 (86) and Microsoft Excel software were utilized for functional calculations, analyses, and graphical visualization of the results.

3.2. Methods

3.2.1. Cultivation of *Leishmania major* isolates

Before removing the *Leishmania* major strains, LRV+ and LRV-, from the -80 ˚C freezer, a liquid complete media was prepared in the specified proportions: 10% FBS, 1% Penicillin-Streptomycin (Penicillin, 10,000 units/ml – Streptomycin, 10 mg/ml), 0.1% Gentamicin, and RPMI medium 1640 with 25 mM L-glutamine. This complete medium, supplemented with 10% FBS, was then referred as "10% FBS RPMI". To enhance cell survival, it is suggested to initiate the culture with 15% FBS RPMI with the same antibiotic concentrations when establishing new cell lines for *Leishmania* species. Once the growth curve stabilizes, the culture can be continued with 10% FBS containing RPMI. The culture medium was prepared in 50 mL Falcon tubes, considering the amount required for the next 2-3 weeks, and ensuring that the complete media were always fresh. The prepared media were stored at +4˚C.

Initially, thawed 10^6/ ml *Leishmania* isolates were mixed with a small amount of culture medium before complete dissolution to minimize the impact of DMSO on the cells. T25 Cell Culture Flasks were used for the culture. Initially, 3-4 ml of 15% FBS RPMI medium was added, and daily microscopic observations were made to observe the viable promastigotes. When the cell count reached a certain threshold, additional 2 ml of fresh complete medium were added until the culture volume reached 10 ml.

3.2.1.1. Maintenance of *Leishmania* culture

Basically, the alteration of the colour of RPMI medium from pink to yellow indicates the passage time of the cell culture. In this step, the culture in the T25 cell

culture flask was transferred to a 15 ml Falcon tube using a serological pipette. The tube was then centrifuged at 1000 xg for 10 minutes. The cells formed a pellet at the bottom of the tube, while the supernatant contained the culture medium. The supernatant was aspirated using a serological pipette, and 1 ml of complete medium was added. Serial dilutions (1:10, 1:100, and 1:1000, depending on the cell density) were prepared in vials using the culture medium, and the pellet from the dissolved culture was diluted in the 1 ml medium. For example, if dilutions were made up to 1:100, 10 µl of the 1:100 diluted sample (dilution factor, DF is 100.) was added to both sides of the counting chamber, known as Thoma chamber or hemocytometer. The counting procedure was performed using the counting technique of the Thoma chamber under a microscope. Since a Thoma chamber has two counting areas, two counts were performed for each culture sample, and the average of the counts was calculated. Based on the counting results, the cell count was determined as follows.

$$Cell\ Count\ =\ X\ *\ 10\text{^}4\ *DF$$

X: Number of cells counted in the Thoma chamber
10^4: Thoma Chamber factor associated with volume
DF: Dilution factor

After determining the cell count, the culture is completed by diluting it with fresh medium to achieve the desired concentration of 500,000 or $1*10^6$ cells/ml.

3.2.1.2. Growth curve of *Leishmania isolates*

To determine the culture stage of *Leishmania major* LRV+ and LRV- strains, the cell line had to be passaged several times to remove the debris. After each passage, cells could be counted under the microscope using a hemocytometer aiming at a concentration of $1*10^6$ cells/ml in 10% FBS RPMI medium. The measurement results can be used to create a line graph using Excel or GraphPad software.

3.2.1.3. Freezing and stocking of isolates of *Leishmania spp.*

For the maintenance of the laboratory work, when excessive production was achieved in the established cell line, the cells were preserved by freezing to obtain fresh isolates for subsequent experiments. The solution required for storing *Leishmania* strains was prepared as follows: a mixture of 35% RPMI, 15% DMSO, and 50% FBS. The solution was divided into 2 ml cryotubes, with a cell concentration of $10*10^6$ cells/ml. The cryotubes were then stored for 1 hour at +4 ˚C, 4 hours at -20 ˚C, and subsequently at -80 ˚C for long-term storage.

3.2.2. *Aspergillus* species on malt extract agar

3.2.2.1. Preparation of malt extract agar

A 1L Erlenmeyer flask was used for preparing 1L of malt extract agar (MEA). 30g of malt extract and 16 g of agar were weighed and added to the Erlenmeyer flask. Millipore Milli-Q distilled water was then added to the flask, ensuring that the total volume did not exceed 1L. The mixture was thoroughly dissolved, and the volume was adjusted to 1L. Sterilization was typically performed by subjecting the medium to a pressure of 1.5 atmospheres at 121°C for 15 minutes. This sterilization protocol is commonly used for liquid substances and for sterilizing MEA medium.a

3.2.2.2. Preparation of malt extract agar petri dishes

After the autoclaved malt extract agar (MEA) cooled down, 25 ml of the liquid medium was poured into each petri dish. The pouring process was carried out using serological pipettes or sterile Falcon tubes. This procedure was performed in a sterile environment, such as a Class II laminar flow cabinet. Approximately 40 petri dishes were filled with malt extract agar from the 1 L medium. The plates were left completely dry, which usually took an hour. Since there were no antibiotics in the medium, the plates were stored at room temperature. Before using the MEA plates, a

waiting period of 2-3 days was required. This waiting period facilitated the identification and removal of any contamination or unwanted growth in the medium.

3.2.2.3. Cultivation of *Aspergillus* species on MEA plate

The TUCIM (2874, 3508, 3616, and 3766) and Uşak6 samples stored in -80˚C freezer were prepared as agar plugs and stored in a 30% glycerol solution. Using a sterile loop, the spore-bearing surfaces of the agar plugs were placed in contact with the fresh MEA plates. The plates were then incubated at 25˚C and monitored daily, with photographs taken to document the progression. Once the entire plate was covered with spores and mycelium, all the plates were sealed with parafilm and stored at +4 ˚C for future use.

3.2.2.4. Aspergillus spore suspension assay with Tween80 protocol

To prepare 0.1% of Tween80 solution, physiological saline solution (SF) was used in this study. In the first experiment, 500 µl of Tween80 was added to 500 ml of SF. The solution was then sterilized in an autoclave.

After growing of the *Aspergillus* strains, the MEA plates with high number of spores were selected at this stage. Approximately 10 ml of 0.1% Tween80 solution was added to the MEA plate containing the spores for their suspension. It was important to collect only the spores without touching the mycelium. More than one 2 ml collection tubes were used for each plate. The Tween80 solution containing spores on the plate was transferred to the tubes using a syringe. The tubes were centrifuged at 12,500 x g for 15 minutes, without filling them up to the brim (to prevent splashing). After centrifugation, the supernatant was discarded, and the Tween80-spore solution was added to the pellet obtained from the centrifugation until no Tween80-spore solution remained on the MEA plate, and the process was repeated.

In the final step, the remaining pellet was washed with double-distilled water (autoclaved MilliQ distilled water). To remove Tween80 detergent from the pellet-

containing tubes, 1 ml of distilled water was added, followed by continuous pipetting. Then, the tubes were centrifuged again at 12,500 x g for 15 minutes, and the pellet should remain at the bottom. (The washing process should continue until the foaming caused by the Tween80 solution ceases.) During the washing process, the pellet-containing tubes were combined to have two tubes. This process was repeated per plate according to the need.

3.2.2.5. Counting of *Aspergillus* spores with hemocytometer

For the *Aspergillus* strains subjected to the washing process, double-distilled water was added to fully dissolve the spores, resulting in a total volume of 1 ml. Serial dilutions were then prepared at ratios of 1:10, 1:100, and 1:1000. Spore counting was conducted using a Thoma chamber, like counting cells with a hemocytometer. 10 µl of diluted spore suspension samples were added to both sides of the Thoma chamber, and the counting was performed twice. The spore counting equation described in the "3.2.1.1. Passaging and counting *leishmania* culture" section was applied to the spore counting process.

3.2.2.6. Stocking of *Aspergillus* species

3.2.2.6.1. Stocking of *Aspergillus* species on MEA plates with agar plug assay

After the completion of the growth stages, the *Aspergillus* species were transferred to a +4°C refrigerator by covering the sporulated MEA plates with parafilm. Using the back of a 1 ml pipette tip, round sections containing sporulated agar were aseptically taken and transferred to 2 ml collection tubes using a sterilized loop. To preserve the samples, a 30% glycerol solution was added to the tubes, and they were stored at -80°C.

3.2.2.6.2. Stocking of *Aspergillus* species with spore suspension solution

After counting the spores, a dilution was prepared for the spore suspension solution with a final concentration of $1x10^6$ spores/mL . This dilution was achieved by mixing the spores with a 30% glycerol solution, resulting in a final concentration of $25x10^6$ spores in the solution. The spore suspension solutions were prepared in 2 mL collection tubes, with 1 mL containing $25x10^6$ spores. These spore stocks were stored at -80°C for preservation. A set of spore stocks was also tested by inoculating them onto MEA plates to verify their viability and growth.

3.2.3. Obtaining class1 hydrophobins of *Aspergillus spp.* spores using trifluoroacetic acid

3.2.3.1. Treatment with TFA compound

For each *Aspergillus* species, the pellets obtained without spore counting using the Tween80 protocol were supplemented with an average of 400 µl of trifluoroacetic acid (TFA), depending on the density of the pellets. The pellets were dissolved in TFA by careful pipetting. If needed, additional TFA could be added based on the solubility of the spores. Then, the samples were sonicated in a water bath for 60 minutes, ensuring that the water level was below the caps of the 2ml centrifuge tubes containing the samples. After sonication, the samples were centrifuged at 12,500 x g for 30 minutes. The resulting supernatant was transferred to a new tube and left in a chemical hood until the TFA completely evaporated. Nitrogen was used to expedite the evaporation process. The tops of the tubes were covered with parafilm, and small holes were made in the parafilm. It is important to label the hood with a warning indicating the evaporation of TFA. Complete evaporation of TFA took 4 to 5 days. Once the TFA had evaporated completely, the samples were either stored dry or wet with PBS at +4°C. This process was repeated for each tube obtained from the Tween80 protocol, as needed.

3.2.3.2. Dissolved in PBS solution

Depending on the density of the samples treated with TFA, some may have visible precipitates while others may appear clear in tubes. 200-300 µl of PBS was added to the samples and vortexed. Subsequently, the samples were centrifuged again at a minimum of 12,500 x g for 15-20 minutes. This step allowed hydrophobins to remain in the supernatant with PBS, while debris was removed by pelleting. The supernatants containing the samples were collected and stored at +4°C.

3.2.4. *Aspergillus* species on different liquid cultures

3.2.4.1. Preparation of malt extract medium

To meet the requirements of each *Aspergillus* strain, it is necessary to prepare Broth with a total volume of 500 ml, considering the use of 50-100 ml for each strain. For the 500 ml solution, 15 grams of malt extract was measured using an analytical balance in a weighing boat. Subsequently, it was poured into a 1 L glass bottle, and Milli-Q distilled water was added without reaching the full 500 ml. A magnetic fish was placed inside the bottle, and with the combination of heat and a magnetic stirrer, the solution was allowed to homogenize. Afterwards, the magnetic fish was removed, and the solution was completed to a volume of 500 ml using the same solvent. The autoclaving process was performed following the liquid protocol, and the solution was stored at room temperature.

3.2.4.2. Preparation of zinc-limiting basal medium AMMH

The zinc-limiting Basal Medium AMMH, also known as *Aspergillus* Minimal Medium (AMM) without a nitrogen source, was prepared by supplementing it with 1 ml of Hunter's trace-element solution containing zinc sulfate. The necessary ingredients for preparing AMM in a 1 L bottle were given in Table 9. After adding the specified amounts of these ingredients, a magnetic fish was used with a magnetic stirrer to facilitate dissolution while adjusting the pH to 6.5 using NaOH. Once the pH

meter was calibrated, pH was adjusted during the solution was stirred, ensuring the even distribution of the added NaOH within a short period of time (87).

Table 9. The quantities of chemical materials required for preparing 1 L of *Aspergillus* Minimal Medium (AMM) without a nitrogen source.

Amount of Chemicals (per L)	**Chemicals**
10 g	Dextrose (D-glucose)
0,52 g	Magnesium Sulfate Heptahydrate (MgSO4.7H2O)
0,52 g	Potassium Chloride (KCl)
1,52 g	Potassium Phosphate Dibasic (KH2PO4)

A total volume of 25-50 mL of Hunter's trace elements solution without zinc sulfate was prepared for 1 L of AMM, with only 1 mL being used (Table 10). The solution was then placed on a magnetic stirrer to facilitate dissolution, and KOH was added to adjust the pH to 6.5.

Table 10. The necessary materials for preparing Hunter's trace elements solution without zinc sulfate are listed.

Amount of Chemicals (per L)	**Chemicals**
11 g	Boric Acid (H3BO3)
5 g	Manganese (II) Chloride ($MnCl_2.4H_2O$)
5 g	Iron (II) Sulfate Heptahydrate ($FeSO_4.7H_2O$)
1.6 g	Cobalt (II) Chloride ($CoCl_2.\ H_2O$)
1.6 g	Copper Sulfate Pentahydrate ($CuSO_4.5H_2O$)
1.1 g	Ammonium Molybdate Tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$)
50 g	Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA)

3.2.4.3. Cultivation of *Aspergillus s*pecies on aspergillus minimal and malt extract liquid medium

Spore suspension stocks were prepared at a high concentration in 25 ml cultures. To ensure a 1/10 ratio for sufficient aeration, 250 ml Erlen flasks were used for each 25 ml culture. Each Erlen flask was labeled for a specific *Aspergillus* species and was intended to be stored for future use in its corresponding liquid culture. The Erlen flask caps were covered with double 4-layered aluminum foil. *Aspergillus* Minimal Medium and Malt Extract Liquid Medium were prepared in 10 Erlen flasks, with 5 flasks for

each medium, and each flask containing 24 ml of medium. Two tubes were taken from the stocks stored at -80 ˚C for each of the 5 different *Aspergillus* strains (2874, 3508, 3616, 3766, and Uşak6), and they were inoculated into different culture media. The cultures were incubated at 30 ˚C and 180 rpm (revolutions per minute). Starting from day 0 and continuing until day 7, a total of 8 times, every 24 hours, 2 ml samples were taken from each culture and replaced with fresh medium to maintain the total volume. The collected samples were labeled and centrifuged at 12,500 x g for 15 minutes. The pellets and supernatants were stored separately at -20 ˚C.

3.2.5. Optimization of fungal metabolites and hydrophobins

Several trials were conducted for optimization. Prior to this step, the obtained samples were subjected to BCA assay, followed by XTT assay but revealed a low pH. Therefore, it was deemed appropriate to first adjust the pH to 7 and then proceed with protein evaluations of the obtained samples.

3.2.5.1. Protein detection: BCA assay

Protein concentrations of the class 1 hydrophobin samples obtained after TFA evaporation and the samples obtained from liquid cultures on a daily basis were calculated using Elabscience's BCA Protein Colorimetric Assay Kit protocol (88).

To generate BCA standards, 1 mg of standard powder was added to 1 ml of standard diluent, and dilutions were made at predetermined ratios. The BCA standards were freshly prepared and can be stored at -20°C for up to 3 months. A 96-well flat bottom plate was set up for the assay. Two replicates were performed using protein standards ranging from 0 to 1 mg to establish a correlation coefficient and conduct regression analysis. The unknown test samples were run in triplicate. Since standard protein was diluted with PBS, it served as the blank. The dilution factor varied depending on the estimated protein quantity of the samples and the amount of each sample. However, there were no inconsistencies in this experiment as the values remained within the expected range. Following the kit protocol, 20 µl of standards and

test samples were added to each well of the plate, followed by the addition of 200 µl of BCA working solution prepared at a 1:50 ratio. The BCA solution can be stored at +4°C for 24 hours. The plate was shaken for 20 seconds and then incubated at 37°C. Subsequently, spectrophotometric measurements were taken at 562 nm using a microplate reader (Varioskan) to determine protein concentrations. The protein concentrations of unknown samples were calculated using regression analysis based on the known standards. The analysis focused on determining the days on which metabolites obtained from liquid cultures produced higher levels of protein, and further cell viability studies were conducted with the identified samples.

3.2.5.2. Optimization of pH

The samples collected daily from the malt extract and *Aspergillus* minimal medium liquid cultures were subjected to BCA assay to identify the samples with the highest protein concentrations. These samples were then titrated with KOH using pH indicator strips to adjust the pH to 7. The same procedure was applied to the class 1 hydrophobin solutions obtained from *Aspergillus* spores treated with TFA. After reaching pH 7, the change in volume caused a variation in protein concentration values, requiring repetation of the BCA assay. The results of both assays were shown in the "Results" section.

3.2.6. XTT assay

The Biotium XTT Cell Viability Assay Kit was used for the cell viability test (89). The Activation Reagent and XTT solution were combined in a 1:200 ratio to create the working solution. The kit instructions indicated that 25 or 50 µl of working solution could be added to each well for every 100 µl of sample.

In the XTT assays for fungi hydrophobins and metabolites, as well as the positive control meglumine antimoniate-treated *Leishmania* cultures, the experimental design included 50 µl of *Leishmania* LRV+ or LRV- culture (twice the desired concentration) and 50 µl l of the treating substance (fungi products or meglumine antimoniate) to

make a total of 100 µl in each well. The test samples were performed in triplicate. Next to the test samples, a negative control group was established for each concentration using *Leishmania*'s own medium (completed 10% FBS RPMI medium) treated with the treating substance, and this group was conducted in duplicate. After optimizing the XTT assay with *Leishmania*, the cell count, amount of XTT working solution, target-background absorbance values for spectrophotometric measurement, and measurement times were kept stable throughout the experiment.

3.2.6.1. Optimization of XTT assay protocol and counting of *Leishmania* promastigotes

For the optimization of the XTT assay, the appropriate number of *Leishmania* promastigotes per well was determined to ensure the kit's optimal performance. Then, the amount of required XTT working solution, whether 25 or 50 µl, was assessed. According to the information provided in the kit, measurements could be taken between 2-24 hours after adding XTT, with the recommended duration typically ranging from 2-5 hours. Measurements were taken hourly, including at the 4th hour, to determine the optimal measurement time that yielded the best results. Finally, for spectrophotometric analysis, the absorbance values were measured within the ranges of 450-500 nm for the desired absorbance and 630-690 nm for background subtraction. Absorbance optimization was conducted at 450-475-500 nm and 630-660-690 nm wavelengths. The measurement with the clearest and most consistent value of the absorbance range was accepted as the fit value for future studies.

3.2.6.2. Treatment of positive control as meglumine antimoniate on *Leishmania* species

A 1% DMSO solution was prepared for dissolving the powdered meglumine antimoniate, which served as the positive control, and the same solution was used for serial dilutions. In a 96-well plate, the first column was designated as the blank containing 50 µl of *Leishmania* cell culture and 50 µl of 1% DMSO solution. The remaining columns were treated with meglumine antimoniate at 11 different

concentrations (ranging from 2 to 2048 ug/ml). For the selected concentration of meglumine antimoniate, the well was supplemented with a double concentration. By maintaining a total volume of 100 µl, the desired cell count and drug concentration were achieved through dilution.

In the initial experiment conducted with the positive control, XTT measurements were taken at 24, 48, and 72 hours to determine the day when the *Leishmania* isolates were affected. The hour that yielded the best results was considered suitable for fungi-derived hydrophobins and metabolites as well.

3.2.6.3. Treatment of class I hydrophobin proteins of *Aspergillus* spores on *Leishmania* species

The *Aspergillus* spores treated with TFA were subjected to acid-sample evaporation, followed by reconstitution with PBS. After adjusting the pH of the samples to 7 using KOH, the protein quantities were calculated using the BCA assay method described in the previous steps. The samples, which were stored at +4 °C, were brought to room temperature, and an 8-point stock solution was prepared through a 1:2 serial dilution using PBS. For the test samples, *Leishmania* LRV+ and LRV- cultures were used, while completed RPMI was used as the negative control. Each column of the 96-well plate was planned to represent a different concentration, and the blank, which contained 50 µl of *Leishmania* culture and 50 µl of PBS without any drug, was considered to have 100% cell viability for analysis purposes.

3.2.6.4. Treatment of fungal metabolites with malt extract medium on *Leishmania* species

After the centrifugation of the samples grown in Malt Extract liquid medium to remove mycelium and spores, the supernatant portion was adjusted to pH 7 to determine the protein concentrations of the metabolites it contained. The XTT assay was planned for the day that showed the highest protein production. Through a 1:2 serial dilution with PBS, eight different concentrations of *Leishmania* culture were

prepared using the metabolite-rich medium obtained from the growth of five different *Aspergillus* samples in Malt Extract. XTT assay was performed without removing the medium. The experiment was conducted by combining the blank, test samples, and negative control with different concentrations of metabolites for *Leishmania* LRV+ and LRV- strains, as described in section "3.2.6. XTT Assay".

3.2.6.5. Treatment of metabolites belong to fungi species with *Aspergillus* minimal medium on *Leishmania* species

The metabolites belonging to *Aspergillus* strains grown in *Aspergillus* Minimal Medium (AMM) on the day with the highest protein concentration were used in conjunction with the medium according to the method described in section "3.2.6.4. Treatment of metabolites belong to fungi species with Malt extract medium on *Leishmania* species." Firstly, the experiment was conducted without removing the medium, and then the determined metabolite-containing media were passed through a 0.20 sterile filter and subjected to XTT assay again. The results of both experiments were shared in the "result" section.

3.2.7. Molecular analysis

For molecular analysis, meglumine antimoniate was used for XTT assay with *Leishmania major* LRV+ and LRV- isolates. To determine the best drug concentration, a total of three different drug concentrations were tested: the 50% inhibitory concentration dose, one upper and one lower of it. As mentioned in the XTT assay method, 100 microliter of samples were mixed with 25 µl of XTT solution. Measurements were made at the 72nd hour using the Varioskan device. After the analysis, RNA isolation was performed.

3.2.7.1. Primer optimization

Before proceeding to quantitative PCR, previously used *Leishmania major* DNA was used to check the functionality of the designed primers. Additionally, DNA

isolation was performed from *Leishmania* culture, and protocol optimizations specific to cell count and *Leishmania* species were conducted using DNA samples instead of RNA due to the stability of DNA. GeneMark Tissue & Cell Genomic DNA Purification Kit (90) was used for DNA isolation. After obtaining pure DNA, optimization studies for cell count, DNA quantity, and primer quantity were performed using conventional PCR. According to the optimization results, the reaction was prepared by adding 2 µl of a solution containing 2 µM of Forward Primer, and 2 µM of Reverse Primer. Also, 3.2 µl of 5X Master mix were added. The total amount of DNA added to the reaction was 100 ng. The reaction volume was then brought to 16 µl by adding nuclease-free water.

Since the melting temperatures of the primers were designed to be 60 degrees, the annealing temperature for all samples was planned as 55 °C. The GeneMark PCR Master Mix II (5X) used contains Taq polymerase capable of producing 1 kb product per minute. Since the product sizes were between 100-200 bp, the extension step was planned for 15 seconds. The PCR run condition protocol was as follows: Denaturation step at 95 °C for 3 minutes, followed by 45 cycles of 10 seconds at 95 °C, 10 seconds at 55 °C, and 15 seconds at 72 °C. Finally, a 1-minute extension at 72 °C was sufficient. PCR products were run on a 2% agarose gel at 100 V for 30-40 minutes using electrophoresis.

3.2.7.2. RNA isolation

For RNA isolation, the Bioelegen Total RNA Purification Kit (91) was used, and the protocol was optimized. The *Leishmania* samples were optimized to a cell density of $50x10^6$ cells/ml. As previously mentioned, five samples of 1000 µl each were prepared at a concentration of $10x10^6$ cells/ml. Preliminary steps were added for cell lysis before the start of the method. These steps are as follows:

2-mercaptoethanol chemical was added to the *Leishmania* culture at a ratio of 1000:7. Then, centrifugation was performed at 7000x g for 5 minutes, and the supernatant was removed. The pellets were then treated with 350 µl of lysis buffer

from the Bioelegen RNA kit and a small amount of zinc-ion beat. After incubating for 10 minutes in a beatbeater, centrifugation was performed at 10,000x g for 1 minute. The supernatant was collected and transferred to a new tube, to which 340 µl of 40 mM Tris-20 mM EDTA solution and 10 µl of proteinase K were added. The mixture was then incubated at 56 °C for 15 minutes. During this step, the samples can be incubated for 1-2 hours. The Eppendorf tubes were vortexed every 5-10 minutes. In the next step, the mixture was centrifuged at top speed for three minutes, and the supernatant was collected. Then, 350 µl of 100% ethanol solution that had been kept at -20 °C was added. These steps were part of the sample preparation process before proceeding to *Leishmania* RNA isolation. Subsequently, the common RNA isolation protocol of the Bioelegen Total RNA Purification Kit (91) was continued for all samples. After elution, the samples were placed in ice, and the RNA quantities were measured and noted using a Nanodrop device.

3.2.7.3. cDNA synthesis

The obtained RNA samples were subjected to DNA synthesis using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (92). The total volume was adjusted to 20 µl, with a calculated amount of RNA to include 1000 ng of RNA in the total sample. The entire process was carried out on ice. Instead of the RNase inhibitor solution that was not included in the kit, the same amount of 40 mM EDTA pH 8 was used. The reaction was prepared according to the kit protocol, and the thermal cycler was optimized as specified. After 10 minutes at 25 °C, 100 minutes at 37 °C, 20 minutes at 42 °C, and 5 minutes at 85 °C, the protocol was set to remain at 4 °C. After completing the incubations, the cDNA quantity was measured using a Nanodrop device. The generated cDNA was used within one week.

3.2.7.4. Quantitative PCR

RNA isolation and cDNA synthesis were performed only on the cultures treated with meglumine antimoniate during the incubation stage. Cultures with a concentration of 1 ml at $50x10^6$ cells/ml, both LRV+ and LRV-, were used for the

process. The cDNA values were measured using the Nanodrop. Optimization was carried out to determine the DNA quantity using the optimized PCR conditions. While 100 ng of DNA was used for PCR reactions, a total of 200 ng of cDNA was required when working with cDNA. To verify that the DNA primers produced the same results in both the RNA and cDNA stages, a conventional PCR was performed using cDNA and the results were visualized by running gel electrophoresis with 2% agarose gel. Invitrogen 50 bp DNA ladder was loaded onto the gel alongside the samples. This stage represents the first molecular-level evidence of the presence or absence of the virus in the samples for this thesis.

For the qPCR analysis, the same protocol as the one used for DNA isolation with the 5X Master Mix was followed. The reaction mixture contained 3.2 µl of Master mix, 0.8 µl of Eva Green, 2 µl of a 2 µM Primer Mix, and the specified amount of cDNA, and the reaction volume was adjusted to 16 µl with nuclease-free water. Since cDNA was used instead of DNA, a total of 200 ng of cDNA was added instead of 100 ng of DNA. The PCR conditions followed the conventional PCR protocol, with a melting curve added at the final stage at a temperature change of 0.5 from 65°C to 95°C.

In addition, the LightCycler® 480 SYBR Green I Master kit was used in this experiment. The primer and DNA quantities were kept the same. The total reaction volume was calculated to be 16 µl. A total of 8 µl of 2X Syber Green I master mix was added, followed by 200 ng of cDNA and nuclease-free water to complete the reaction. Real-time qPCR analysis was performed using the same protocol (93).

$$\Delta CT = CT\ gene\ of\ interest - CT\ internal\ control$$
$$\Delta\Delta CT = Treated\ sample\ \Delta CT - Untreated\ sample\ \Delta CT$$
$$2^{-\Delta\Delta CT} = Fold\ change\ of\ gene\ expression$$

Untreated samples and treated Leishmania LRV+ and LRV- cultures at three different concentrations were used to perform RNA and cDNA analysis, and each

sample was subjected to duplicated technical repeats in the qPCR analysis. To determine the fold change in gene expression, the 2^(-ΔΔCT) method was used (94).

The GAPDH gene was used as the housekeeping gene. The *Leishmania major*-specific KMP11 gene was also considered as a housekeeping gene and compared to GAPDH for the analysis. C_T values obtained from the qPCR analysis of TRYR, GP63, and DHFR genes were analyzed to calculate the fold change values.

4. RESULTS

4.1. Cultivation of *Leishmania spp.*

It was observed that the *Leishmania* major LRV+ strain adapted better to the culture conditions compared to *L. major* LRV- strain. Due to a temporary malfunction in the temperature indicator of the 25˚C incubator in the laboratory, that increased the temperature to 27˚C, the LRV- L. major culture could was not expected for a few weeks. However, the LRV+ strain adapted to the altered conditions and returned to normal conditions within one week.

Cell counting was performed using a Thoma chamber before each passage. Leishmania cultures were taken at a concentration of $1x10^6$ cells/ml, and cell counting was performed daily. The mean and standard deviations of the cultured samples, conducted in triplicate, are shown in Figure 13. *L. major* LRV+ and LRV- cultures reached the logarithmic phase on the 2nd day and remained in this phase until the 5th day. After the 5th day, they are presumed to be in the stationary phase. In this in-vitro study, it is estimated that the promastigote form of *Leishmania* was in the procyclic stage until the 5th day, and they were in the metacyclic form between the 5th and 7th days. Additionally, when the two culture isolates were compared, it was observed that the culture including viruses was more adaptive and had a higher reproductive rate compared to the non-viral isolate. The cell count at the end of the logarithmic phase indicated that the viral isolate had 16% more cells compared to the non-viral isolate.

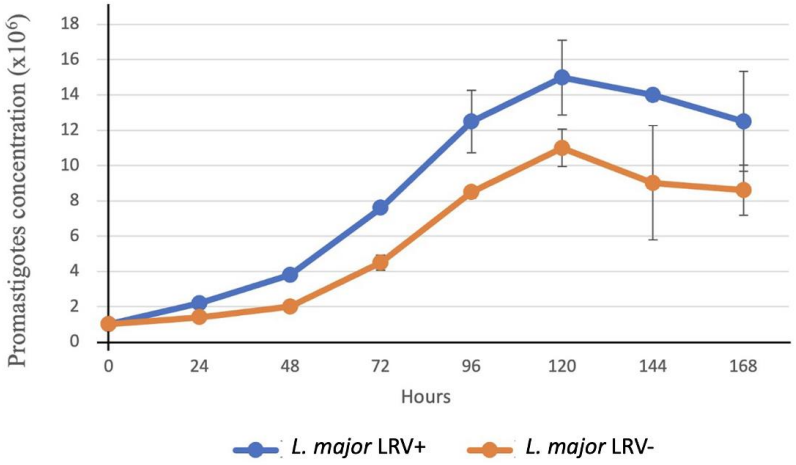

Figure 13. Growth curve graph of *L. major* LRV+ (blue) and LRV- (red) cultures.

Isolates stored at -80˚C in Acibadem University Research Laboratory were thawed according to the protocol to obtain fresh stocks. Subsequently, both test cultures was set to confirm the viability of *Leishmania* cells.

4.2. Cultivation of *Aspergillus spp*.

4.2.1. *Aspergillus species* on malt extract agar

Firstly, malt extract agar (MEA) was prepared in petri dishes according to the protocol. Agar plugs from TUCIM 2874, 3508, 3616, 3766, and Uşak6 samples stored at -80 ˚C in Acıbadem University Research Laboratories were transferred onto fresh malt extract agar. MEA plates were incubated at 25 ˚C in two different conditions: covered with and without parafilm. In the parafilm-covered plates, excessive moisture caused condensation on the lids, resulting in water accumulation over time on the growing fungi. However, the hydrophobic nature of the spores and mycelium allowed the water to flow off without causing any degradation of the samples. Only the uncovered plates were photographed.

The growth of the fungi was observed day by day. Photographs were taken daily using the BioRad ChemiDoc Imaging System. The growth ranges of the *Aspergillus* samples 2874 in Figure 14 and 3616 in Figure 15 from the TUCIM collection are

shown from day1 to day6. Photographs of the 3508, 3766, and Uşak6 samples can be found in Appendix 1.

During the observations, it was noted that for the samples 3616 and Uşak6, multiple inoculations resulted in plates mostly with only mycelium and no sporulation. However, for the samples 2874, 3508, and 3766, no MEA plate was observed without sporulation. It was also observed that the spore structures of the 2874 and 3508 samples appeared smaller compared to other species. It was observed that the mycelial spread of 3616 and Uşak6 proceeded in a more circular manner, while in other species, spores were observed to jump to one corner of the plate and initiate growth from there.

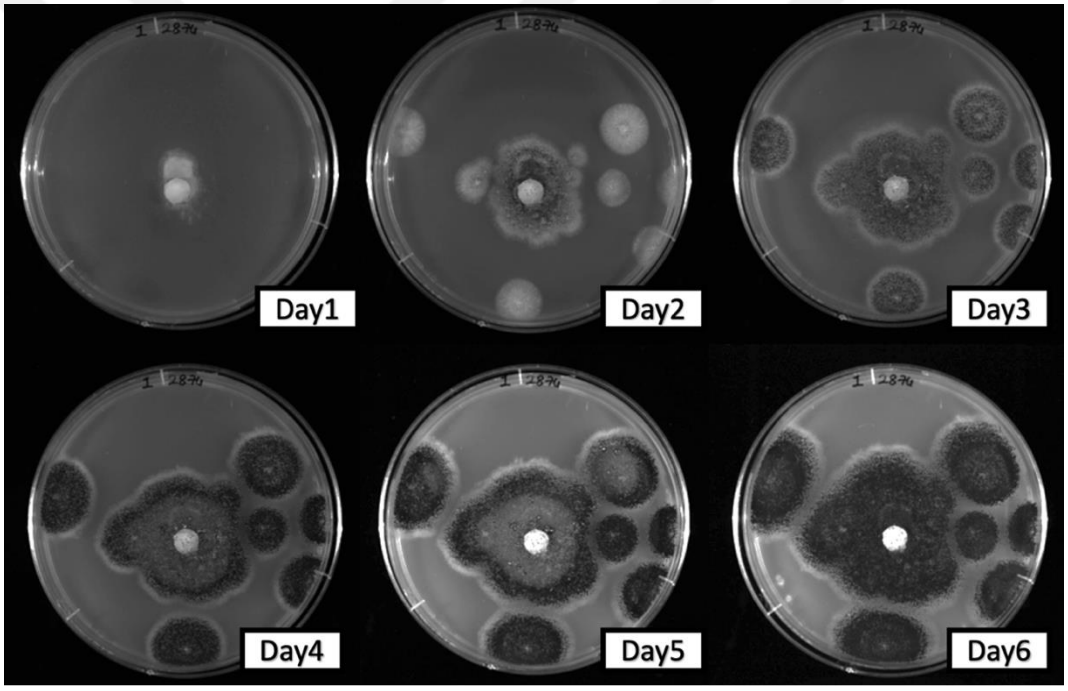

Figure 14. The daily observation of sample 2874 from *Aspergillus* species on malt extract agar.

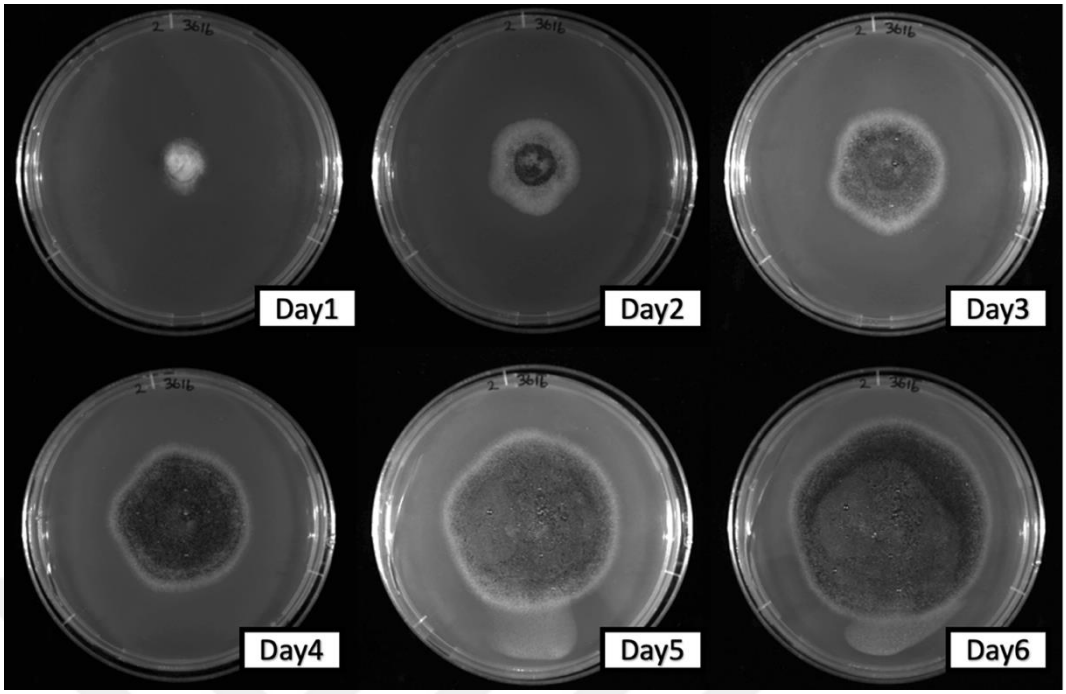

Figure 15. The daily observation of sample 3616 from *Aspergillus* species on malt extract agar.

In addition, the MEA plates were also examined under a microscope. Morphological differences were observed in the mycelial structures at the outer edges of the growing colony. The mycelium exhibited growth both close to the surface of the medium and within the agar. In summary, the extension of mycelium within the culture medium was observed.

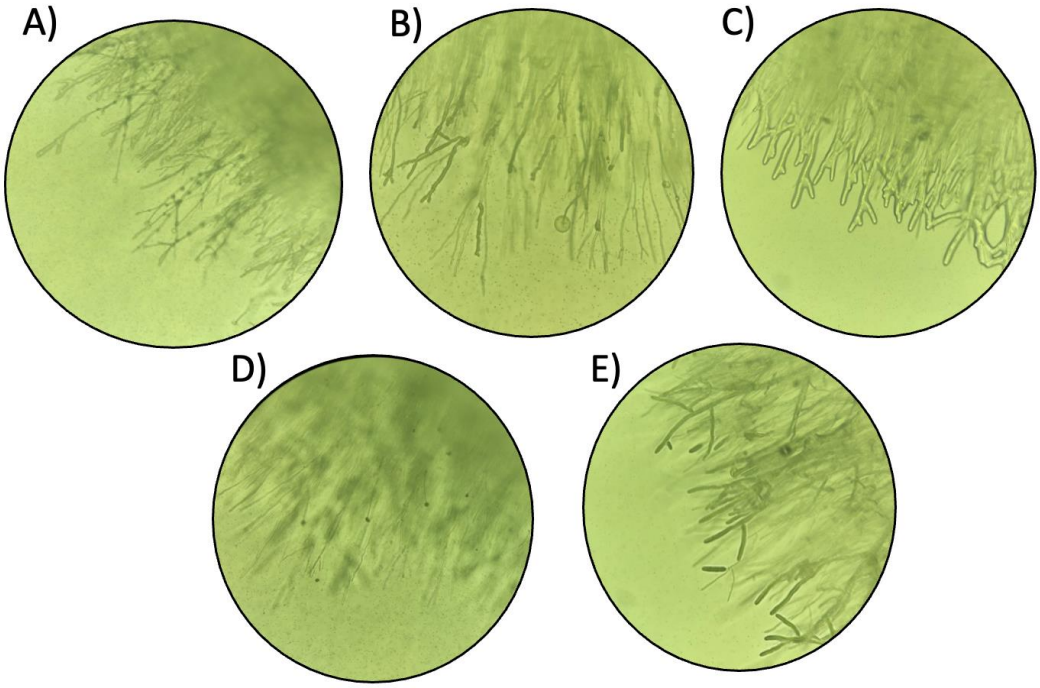

Figure 16. The microscopic images of the fungal samples on malt extract agar are shown sequentially. A) 2874, B) 3508, C) 3616, D) 3766, and E) Uşak6 samples are shown.

The images in Figure 16, except the image C belongs to 3616 sample, were captured using a ZEISS AXIO microscope with an A-Plan 20x/0.45 R objective. The 3616 sample was captured using the same microscope with an N-Achroplan 40x/0.65 R lens. It is worth noting that Uşak6 exhibited morphologically distinct mycelial structures compared to the others.

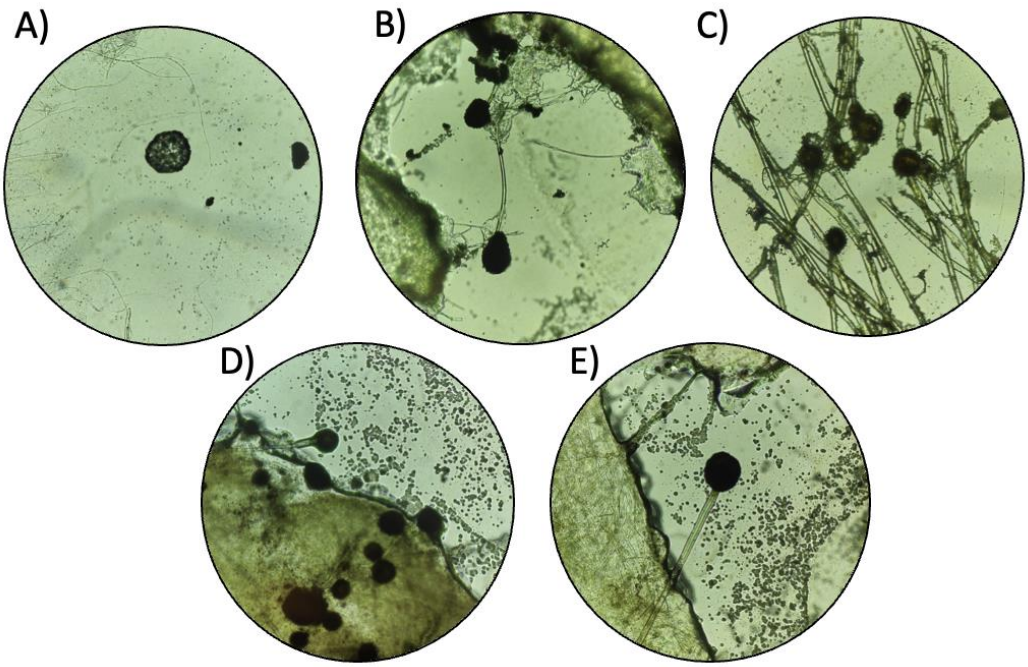

Figure 17. Examination of cross-sections from Aspergillus species on Malt Extract Agar under the microscope. The *Aspergillus* species, namely A) 2874, B) 3508, C) 3616, D) 3766 and E) Uşak6, are shown in sequence.

The growth of the five fungal species on MEA plates at 25 ˚C took approximately 7-10 days. After the growth was completed, some plates were set aside for stock purposes. Agar plugs were prepared with 30% glycerol for stock, following the agar plug protocol. During the procedure, sections of *Aspergillus* species were taken onto a slide and spread in double distilled water to observe mycelial and sporangial structures under the microscope. Some sporangia were seen as bursted during sectioning, with spores dispersed around. The observation was conducted using a ZEISS AXIO microscope with a 40x/0.65 R lens, as shown in Figure 17. No significant differences in sporangia or mycelial structures were observed among the species; rather, structures specific to fungi were more prominent.

4.2.2. Treatment with trifluoroacetic acid on *Aspergillus* spores

Aspergillus samples grown on Malt Extract Agar were collected using the Tween80 protocol, followed by a washing process to obtain spore pellets. Trifluoroacetic acid (TFA) was added to the pellets and incubated in a sonicated water

bath for 60 minutes. After the entire protocol was completed, the samples were left in the hood for approximately 4-5 days until all TFA evaporated. Following the procedure, PBS was added to the samples according to their volume, and another centrifugation was performed to eliminate brown debris. The samples were stored at +4 ˚C.

4.2.3. *Aspergillus* species on malt extract broth

The spores of *Aspergillus* species grown on malt extract agar were collected using the Tween80 protocol. The collected spores were washed with double distilled water, and spore counting was performed using a Thoma chamber. All experiments to be conducted in liquid media were planned with a volume of 25 ml. Therefore, a stock solution with a concentration of $25x10^6$ spores/ml and 30% glycerol was prepared.

Malt Extract Broth was prepared and autoclaved, and 24 ml of the broth was added to 250 ml Erlenmeyer flasks. The identified five *Aspergillus* species, stored at -80 degrees Celsius, were inoculated into the flasks. The flasks were incubated at 30 ˚C and 180 rpm. Cultures were sampled a total of eight times, including day 0 and day 7, with 2 ml of culture collected each day and replaced with fresh malt extract broth. The collected culture samples were stored in separate Eppendorf tubes as supernatant and pellet at -20 ˚C.

During the 7-day observation period, it was observed that the Uşak6 sample formed a mycelial ball of approximately 3-4 cm in size, with the outer surface covered in spores, starting from day 3 (Figure 18). The other samples exhibited homogeneous distribution of mycelial balls of approximately 2-3 millimeters in size. In the case of the 3616 sample, the size of the mycelial balls was slightly larger, while in the 2874 sample, it was smaller compared to the 3766 sample.

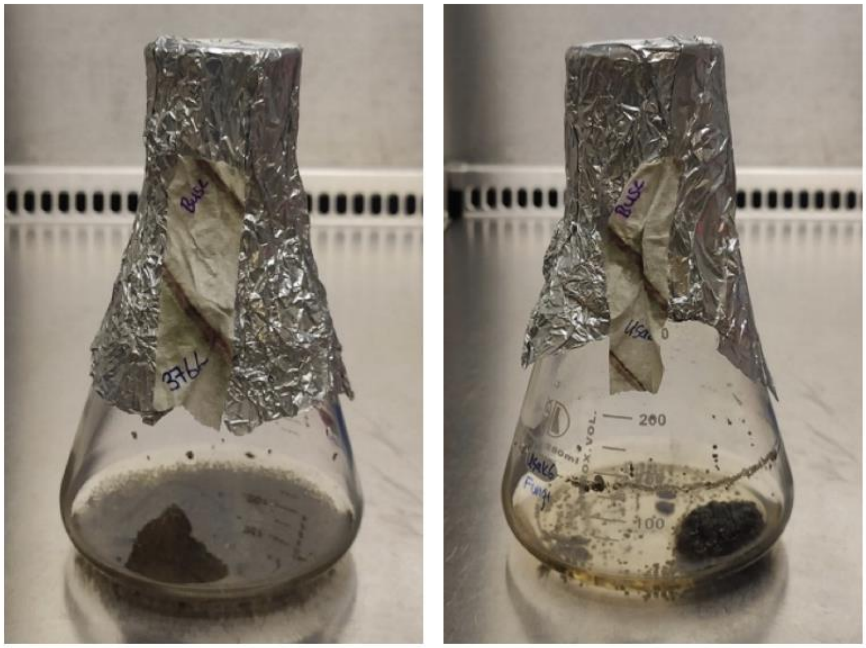

Figure 18. The 3rd day of the Malt Extract culture of Aspergillus species shows the 3766 sample (left) and the Uşak6 sample (right). While the Uşak6 sample exhibited a single large mycelial ball, similar to the 3766 sample, the mycelial balls in the other samples were small and evenly distributed within the culture.

Although the *Aspergillus* samples were distributed homogeneously in the Malt Extract culture, the mycelial balls could not be collected in equal quantities in the sampled cultures. Therefore, weighing procedures were conducted to determine the amount of mycelium and spore production in the daily measurements planned for the experiment. However, due to the unequal collection of samples, a correlation could not be established between the measurements.

On the 7th day, all samples were collected and stored at -20 ˚C. Before removal, mycelial balls were transferred onto slides, and dimensional differences were observed under the microscope. The samples shown in Figure 19 belong to the *Aspergillus* species with the numbers 3508 and 2874. The dimensional differences of the mycelial balls can be observed: 2874 is smaller, while 3508 is larger. The images were observed using the N-Achroplan 5x/0.13 lens on a ZEISS AXIO microscope.

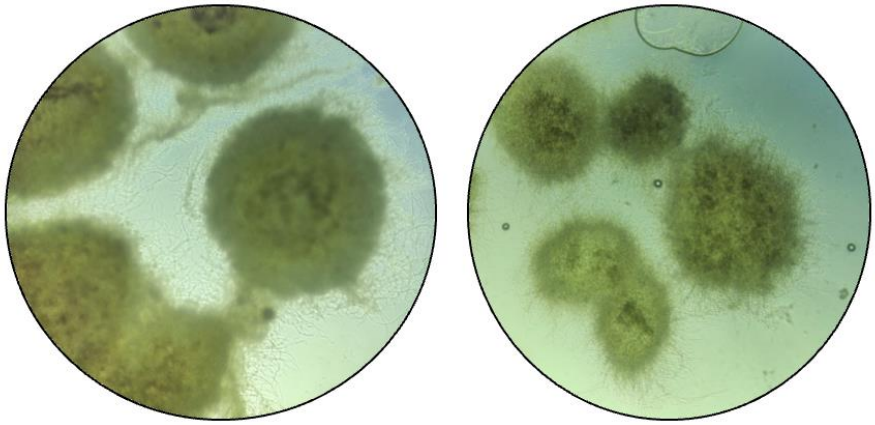

Figure 19. The mycelial balls formed by the Aspergillus species, samples 3508 and 2874, in the Malt Extract liquid culture. *Aspergillus* species 3508 (left) and 2874 (right). A dimensional comparison has been made here. The diameters of the mycelial balls are approximately 2-3 mm for 3508 and 1-2 mm for 2874.

Mycelium and sporangium structures were observed. Microscopic images of the five *Aspergillus* species are provided in Appendix 2.

4.2.4. *Aspergillus* species on *Aspergillus* minimal liquid medium

Stocks prepared at a concentration of 25 x10^6 spores/ml for *Aspergillus* species were combined with *Aspergillus* Minimal Medium (AMM) in Erlenmeyer flasks containing 24 ml each. In the *Aspergillus* cultures incubated at 180 rpm and 30 ˚C, the mycelial balls observed in the *Aspergillus* samples grown in Malt Extract Broth were not observed. Instead, very thin filamentous brown structures were observed in the AMM cultures. Another observation was that sample 3616 had a lighter color compared to the others, as seen in Figure 20. The color of the *Aspergillus* minimal medium is transparent, and the color changes after the addition of *Aspergillus* strains.

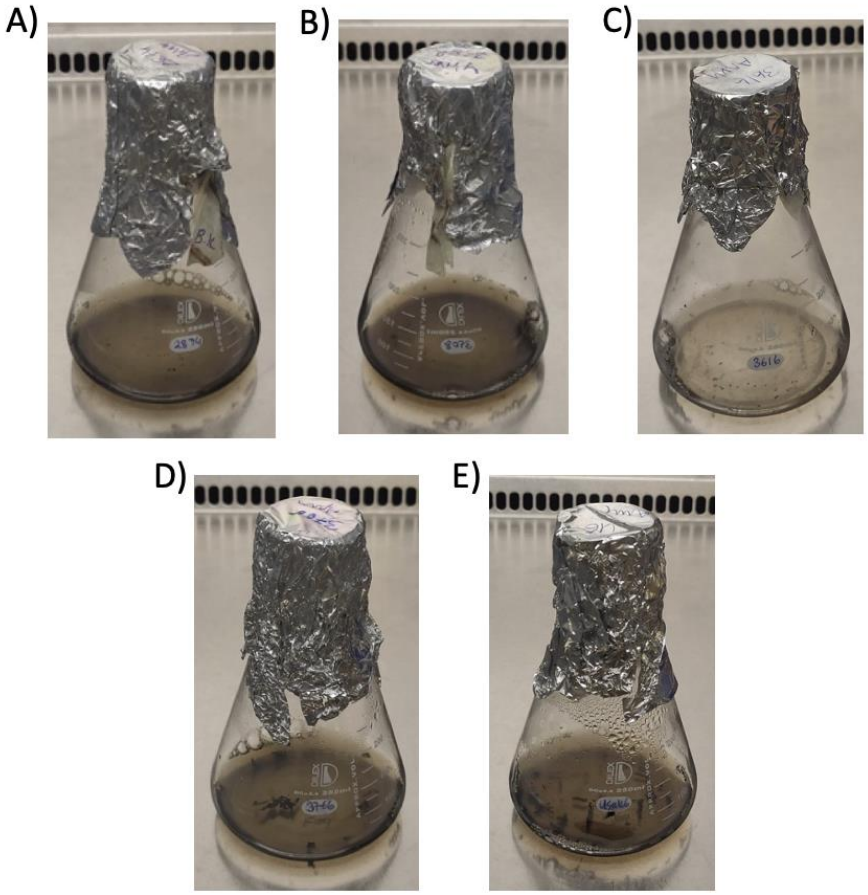

Figure 20. The 4th-day observation of *Aspergillus* species in *Aspergillus* Minimal Liquid Medium (AMM). A) 2874, B) 3508, C) 3616, D) 3766, and E)Uşak6 samples are shown.

Similar to the Malt Extract culture, 2 ml samples were collected daily from the AMM culture and replaced with fresh AMM medium. The collected samples were stored at -20 ˚C. On the 7th day of the AMM culture, before all the cultures were transferred to -20 ˚C, samples were taken from the culture to examine under the microscope on a slide, which would in time contain the filamentous structures. While mostly mycelial structures were observed in the collected samples, some samples like 2874 showed sporangium structures when focusing on the filamentous structures, as seen in Figure 21. This indicates the presence of reproduction in the AMM culture. The mycelial structures of samples 3508, 3616, 3766, and Uşak6 are shown in Appendix 3.

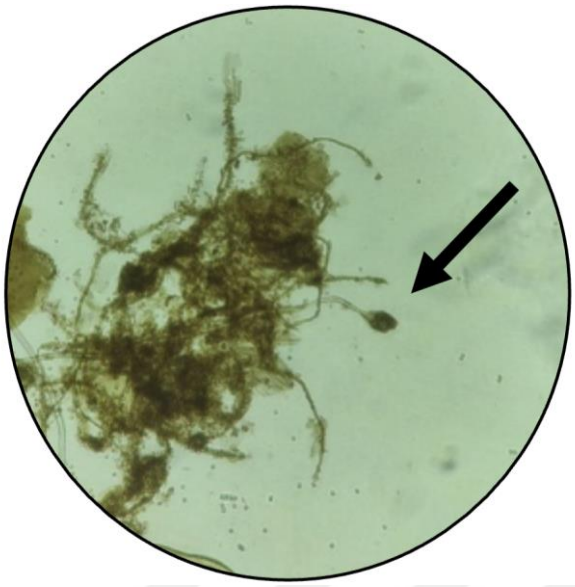

Figure 21. The sporangium of *Aspergillus* sample number 2874 in *Aspergillus* Minimal Liquid Medium.

In the sample 2874, special attention was given to the filamentous brown structures observed in other samples under the microscope. A sporangium was found to be there, which was indicated by a black arrow. It was observed and photographed using a ZEISS AXIO microscope with an A-Plan 20x/0.45 R lens (Figure 21).

4.3. Optimization of the pH of Fungi-based Products and BCA Assay Before Treatment

BCA assay was performed on spore samples treated with TFA and metabolite samples obtained from fungal cultures. In the initial experiment, the samples were diluted without considering the pH values. Reaction was observed with BCA in the TFA-treated samples. The pH of the TFA-treated samples was measured. The pH values of the samples were measured using pH indicator strips, and the samples that underwent the reaction had pH values in the range of 0-1. Subsequently, the pH values of the samples were raised to 7 using KOH followed by the application of BCA assay. The total protein concentration of Class I hydrophobins at pH 7 is shown in Table 11.

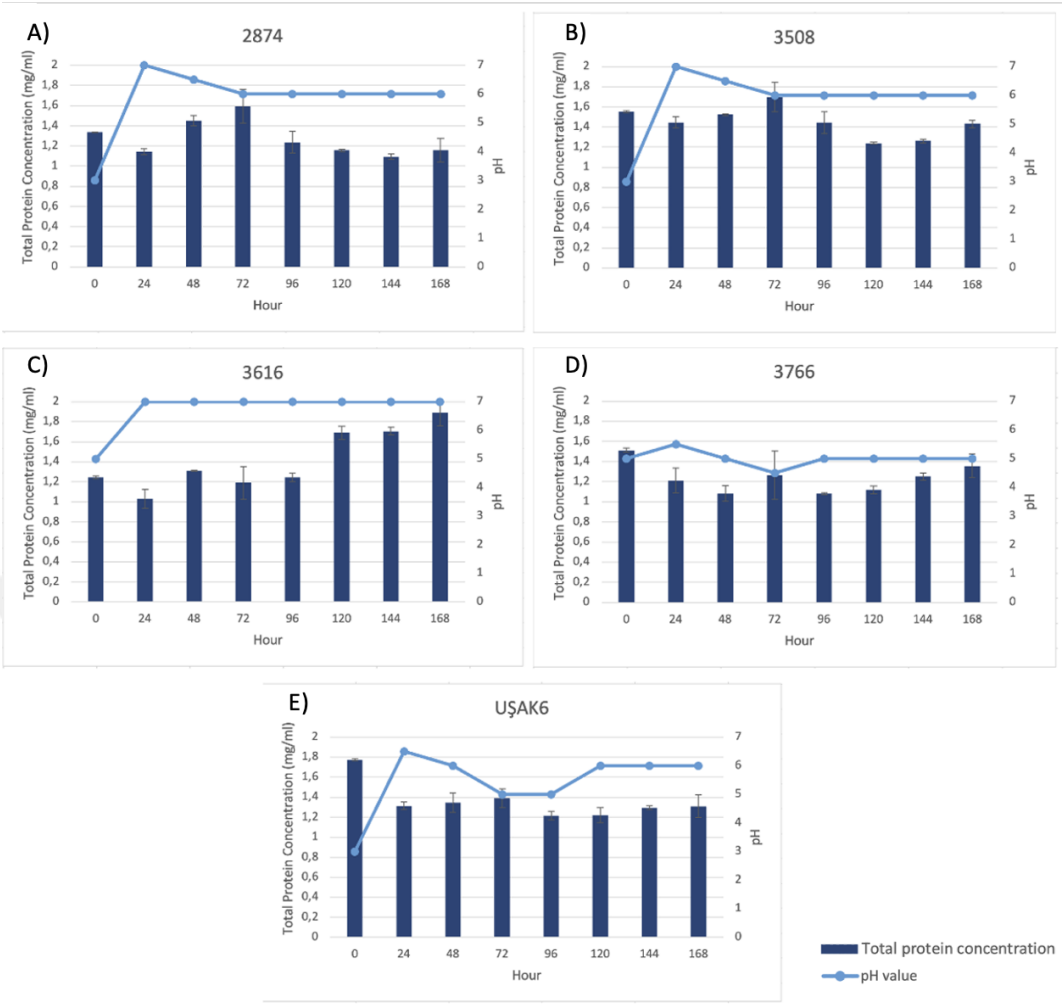

Figure 22. The pH and total protein concentration values of metabolite samples collected from *Aspergillus* species in *Aspergillus* minimal medium (AMM) over a period of 7 days. The *Aspergillus* samples a) 2874, b) 3508, c) 3616, d) 3766, and e) Uşak6 are shown. The pH and protein concentration changes of the metabolites between 0-168 hours are displayed. The columns represent the total protein concentration, while the lines represent the pH values.

The graphs illustrating the pH values and total protein concentration of *Aspergillus* strains grown in AMM culture are shown in Figure 22. Samples were collected every 24 hours within the 0-168. hour range. According to the graphs, the initial pH values of the 3616 and 3766 samples are 5, while the pH values of the other samples are 3. The pH value of AMM medium is 7. On the final day, the pH values of 3616 and 3766 samples are 7 and 5, respectively, while the pH of the other *Aspergillus* samples, which initially had a pH of 3, stabilizes at pH 6 after the 4th or 5th day.

On Day0 assessment, *Aspergillus* samples contained a mixture of spores and AMM medium. The protein concentration here was 1.2-1.8 mg/ml, attributable to AMM. A protein production trend was observed in the 2874, 3508, and Uşak6 samples at 24-48-72 hours. In the case of the 3616 *Aspergillus* sample, the total protein value, starting at 1.2 mg/ml on day 0, gradually increased until the 7th day. Except for the 3616 *Aspergillus* strain, the highest total protein is observed on the 3rd day in the other samples. Metabolite samples from the 3616 sample on the 6th or 7th day, and from the other samples on the 3rd day, were selected for testing on *Leishmania* isolates.

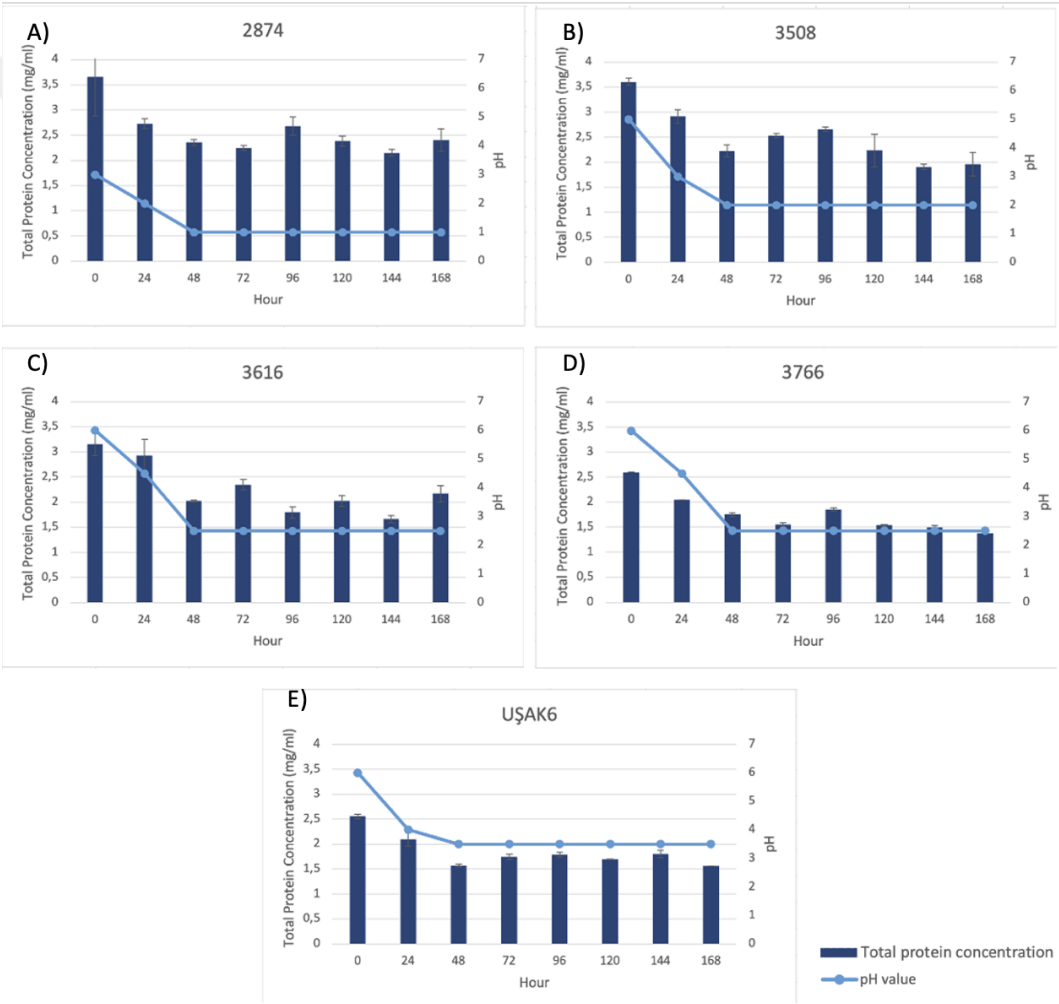

Figure 23. The changes in total protein concentration and pH values of the Aspergillus samples cultured in Malt Extract Broth are shown day by day. The samples are denoted as a) 2874, b) 3508, c) 3616, d) 3766, and e) Uşak6. The column graph represents the total protein concentration, while the line graph represents the pH values.

The pH and protein concentration results of the *Aspergillus* species cultured in Malt Extract Broth were shown in Figure 23. The observed pH values initially start at pH 6 for 3616, 3766, and Uşak6, while the 3508 sample begins at pH 5. In the case of the 2874 sample, the pH value started even lower at pH 2, but decreased to pH 1 during high production. The pH values of the samples decreased by 2-3 units in the first two days and then were stabilized after 48 hours. The pH value of malt extract medium was 5.5.

The protein values on the first day were the highest, as measured in the presence of malt extract in the culture medium. When comparing the species, the highest total protein values above 2 mg/ml were observed consistently in the 2874 sample, while the 3766 and Uşak6 samples showed values ranging from 1.5 to 2 mg/ml. In all samples, the total protein concentration reached highest level on the 4th day.

The samples with the highest protein production on specific days were selected, and their pH values were determined using pH indicator strips. The acidic pH values were adjusted to 7 using KOH. Dilutions were made based on the spore quantities in the samples to ensure they fell within the range of the BCA Assay. During the pH adjustment to pH 7, dilutions were performed, resulting in changes in the protein concentration ratios. These samples, along with others listed in Table 11, were used in the XTT Assay to test their effects on *Leishmania* LRV+ and LRV- cultures.

Table 11. The results of the BCA Assay performed at pH 7 on the *Aspergillus* species cultured in *Aspergillus* Minimal Medium, Malt Extract Broth, and samples treated with TFA.

Medium	Day	Species	pH	Protein Concentration (mg/ml)
AMM	3	2874	7	1,4618938
AMM	3	3508	7	1,662209347
AMM	6	3616	7	2,606501013
AMM	3	3766	7	1,57229548
AMM	3	Uşak6	7	2,045802923
ME	4	2874	7	1,094885804
ME	4	3508	7	0,965622507
ME	4	3616	7	0,835223854
ME	4	3766	7	1,168262865

Table 11. (Cont'd) The results of the BCA Assay performed at pH 7 on the *Aspergillus* species cultured in *Aspergillus* Minimal Medium, Malt Extract Broth, and samples treated with TFA.

Medium	Day	Species	pH	Protein Concentration (mg/ml)
ME	4	Uşak6	7	1,094054415
TFA	none	2874	7	0,933845186
TFA	none	3508	7	0,511265435
TFA	none	3616	7	0,488535685
TFA	none	3766	7	0,135930617
TFA	none	uşak6	7	2,177617106

4.4. Optimization of XTT Assay

An optimization study was conducted to establish the protocol for the XTT Assay, which involved optimizing the number of *Leishmania* cells, the amount of XTT working solution, and the time points for measurements. As the quantity of *Leishmania major* LRV+ culture was higher during the planned experiment, the optimization study was performed using the LRV+ culture.

The range of *Leishmania* cell concentrations tested was between 62,500 and 32 $x10^6$ cells/ml. The Biotium XTT Assay protocol is provided in Appendix 4. According to the protocol, either 25 or 50 µl of XTT working solution could be used for 100 µl of the initial trial, the start time was considered as 0 hours when *Leishmania* was added to the 96-well flat-bottom plate, and XTT was added at the 68th hour. Measurements were made at every hour starting from the 4th hour. Figure 24 illustrates the analysis for different *Leishmania* cell concentrations using 50 µl of XTT working solution, while Figure 25 shows the analysis using 25 µl of XTT working solution. The graphs were plotted using Microsoft Excel, and the standard deviation values are indicated on the graphs.

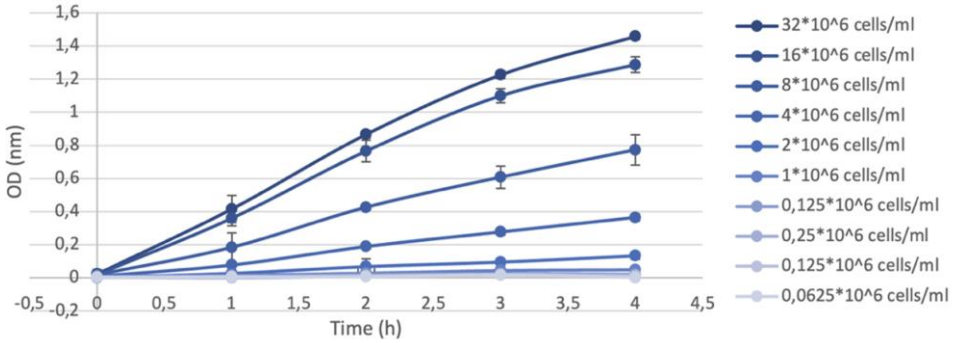

Figure 24. XTT Assay Optimization for *Leishmania major* LRV+ with 50 µl XTT Working Solution.

Upon examining Figure 24 and Figure 25, it was determined that the optimal measurement time after adding XTT was the 4th hour. Regarding the amount of working solution, there was no significant difference in the OD values between the 25 µl and 50 µl samples at the 4th hour. Therefore, it was decided to proceed with 25 µl of XTT working solution. As for the *Leishmania* cell count, it was decided to use a concentration of 10 x10^6 cells/ml for all XTT assay time points, considering both the range of possible OD values in the assays and the circulation of the *Leishmania* culture.

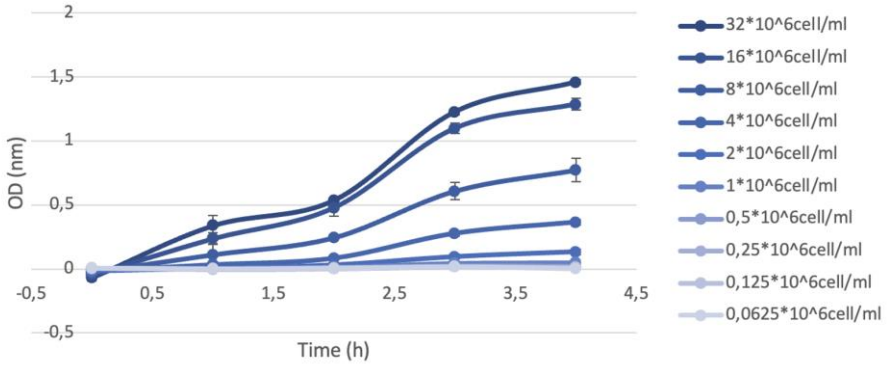

Figure 25. XTT Assay Optimization for *Leishmania major* LRV+ with 25 µl XTT Working Solution.

The XTT protocol specifies that sample measurements should be taken in the range of 450-500 nm, while the background measurement for subtracting XTT background should be taken between 630-690 nm. In the initial optimization, measurements were taken at 450, 475, 500, 630, 660, and 690 nm absorbance values

using the Varioskan Flash Spectral Scanning Multimode Reader. Based on the measurement results, the most reasonable values for samples were found at 450 nm, while the background measurement was found at 630 nm. These two measurements were taken in subsequent XTT assay experiments.

4.5. Treatment of meglumine antimoniate and hydrophobins and metabolites from *Aspergillus spp*. on *Leishmania* isolates

4.5.1. Treatment of meglumine antimoniate on *Leishmania spp.*

The samples were treated with Meglumine Antimoniate in a concentration range of 2-2048 µg/ml, with a cell concentration of $10x10^6$cells/ml of *Leishmania*. The samples treated with only the drug-containing medium were performed with two replicates, while the samples treated with both the drug-containing medium and *Leishmania* had three replicates. The Meglumine Antimoniate solution was prepared by dissolving 50 µl in 1% DMSO, diluted with 10% FBS Complete RPMI medium or a $20x10^6$cells/ml concentration LRV+ *Leishmania* isolate. A total volume of 100 µl was added to each well.

Two sets of samples were prepared, and XTT was added at 44 hours and 68 hours. The absorbance values were read using the Varioskan device at 48 hours and 72 hours. The IC50 values were determined by taking the logarithm of the concentrations, considering the untreated blank sample as 100% viability, and determining the inhibitory concentration of meglumine antimoniate based on the percentage of cell viability. The OD values measured at 48 hours and 72 hours were used to plot the graphs shown in Figure 26. The graphs were generated using GraphPad Prism 9.

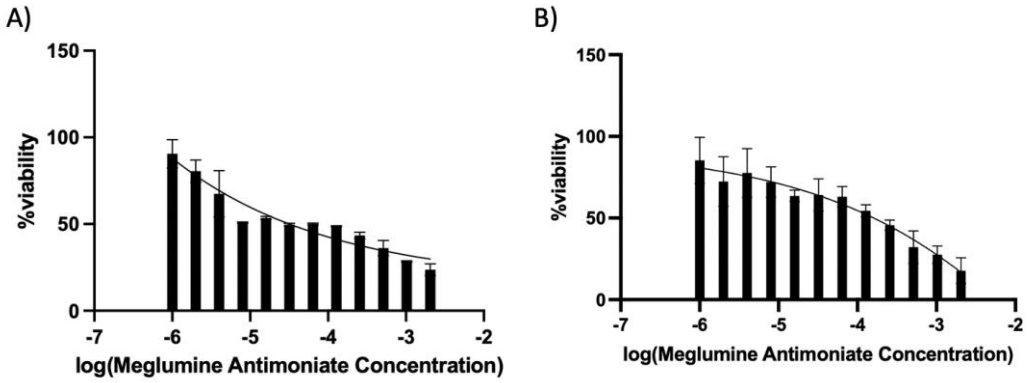

Figure 26. The cell viability of *Leishmania major* LRV+ treated with Meglumine Antimoniate (at A) 48 hours and B) 72 hours).

4.5.2. Treatment of Class I hydrophobins of *Aspergillus* spores on *Leishmania* spp.

The protein concentrations of Class I hydrophobins derived from *Aspergillus* spores treated with TFA were determined using BCA Assay at pH 7. In this experiment, a total of 8 different concentrations of each *Aspergillus* hydrophobin sample were tested on *Leishmania major* LRV+ and LRV- strains. The tested total protein concentration ranges for the *Aspergillus* samples are as follows: Sample 2874 ranged from 7.296 to 933.845 µg/ml, Sample 3508 ranged from 3.994 to 511.265 µg/ml, Sample 3616 ranged from 3.817 to 488.536 µg/ml, Sample 3766 ranged from 1.062 to 135.931 µg/ml, and Sample Uşak6 ranged from 17.013 to 2177.617 µg/ml. Upon completion of XTT Assay optimization, the experiment was finalized. The cell viability analyses are shown in Figure 27. The graphs were created using Graphpad Prism 9.

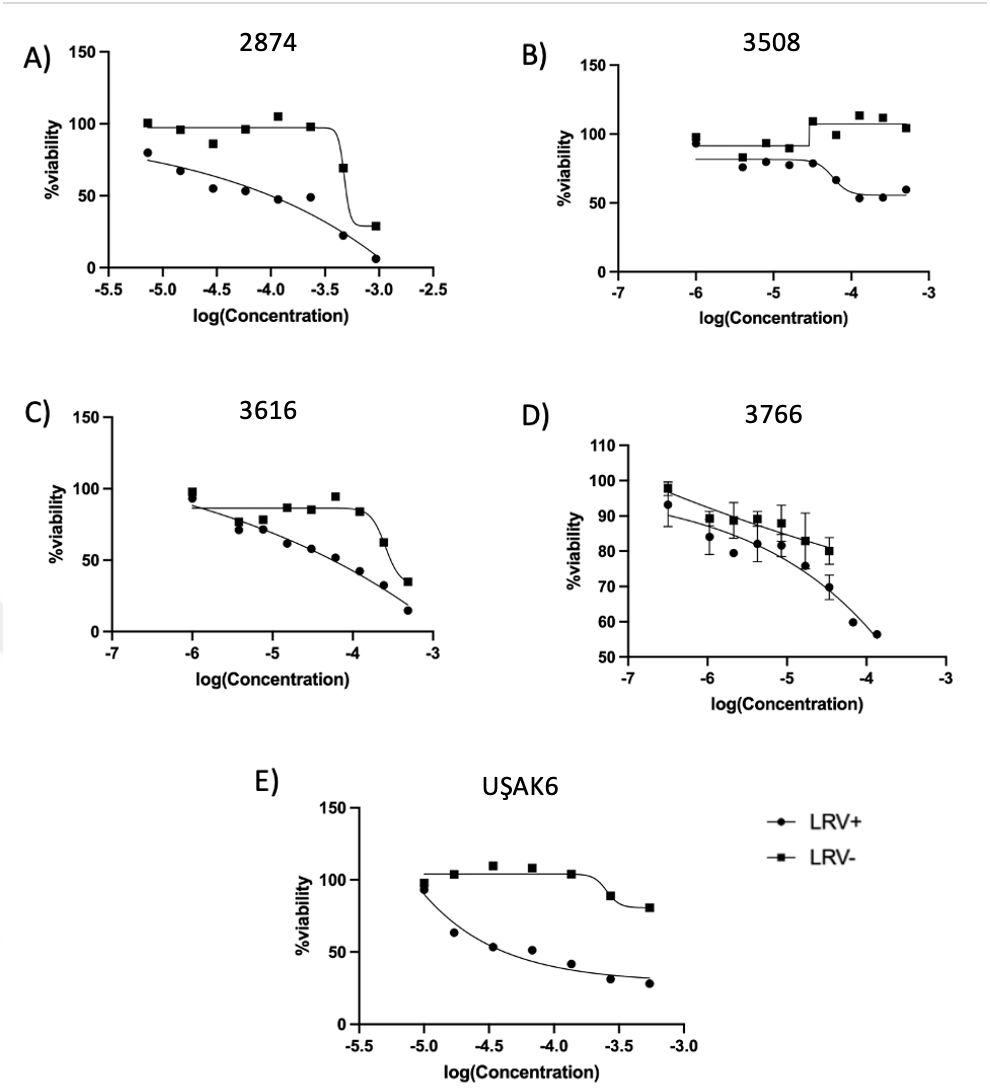

Figure 27. The cell viability of *Leishmania major* LRV+ and LRV- isolates in the presence of Class I hydrophobins from *Aspergillus* species. Viability graphs of these samples are shown as A) 2874, B) 3508, C) 3616, D) 3766, and E) Uşak6.

Results showed that, except for sample 3508, other samples were effective on *Leishmania major* LRV+ isolate. Sample 3508 did not show any effect on neither isolates. The 50% cell viability value for sample 3766 could not be observed. In the case of sample Uşak6, 50% cell viability was observed for LRV+ isolate but not for LRV- isolate. In samples 2874 and 3616, a rapid decrease was observed in the highest two concentrations for the LRV- isolate. However, for the LRV+ isolate, the effect of these samples was not sharp but showed a gradual decrease. It can be stated that the hydrophobin samples were more effective on the LRV+ isolate compared to the LRV- isolate.

4.5.3. Treatment of metabolites of *Aspergillus spp.* with malt extract broth culture on *Leishmania spp.*

The day with the highest protein production by *Aspergillus* strains grown in Malt Extract Broth was determined to be the 4th day using the BCA test. Here, the effects of these metabolites were tested on *Leishmania* isolates in eight different concentrations with a 1:2 dilution. The protein concentration ranges for the *Aspergillus* samples were as follows: sample 2874 ranged from 8.554 to 1094.886 µg/ml, sample 3508 ranged from 7.544 to 965.622µg/ml, sample 3616 ranged from 6.525 to 835.224 µg/ml, sample 3766 ranged from 9.127 to 1168.263 µg/ml, and sample Uşak6 ranged from 8.547 to 1094.054 µg/ml. Cell viability analyses were performed using Excel and plotted using GraphPad Prism 9. Figure 28 shows the effects of metabolites produced by *Aspergillus* in malt extract medium on *Leishmania* isolates.

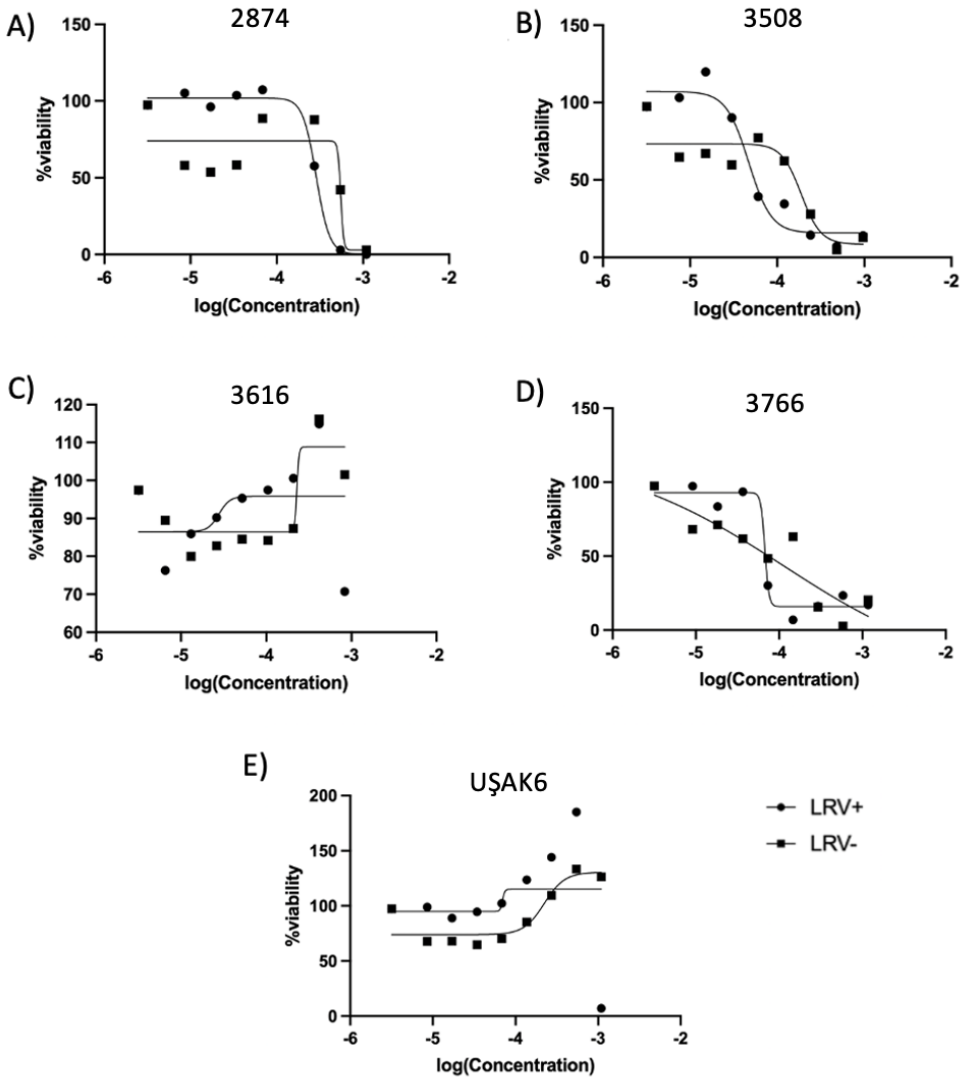

Figure 28. The cell viability of *Leishmania major* LRV+ and LRV- isolates in the presence of metabolites from *Aspergillus* species cultured in malt extract broth. They are respectively determined as A) 2874, B) 3508, C) 3616, D) 3766, and E)Uşak6.

Analysis indicated that the metabolites from samples 3616 and Uşak6 did not have any effects on neither *Leishmania* isolates. However, samples 2874, 3508, and 3766 were found to be effective on both *Leishmania* isolates.

4.5.4. Treatment of metabolites of *Aspergillus spp.* with *Aspergillus* minimal medium culture on *Leishmania spp*.

It was determined that most *Aspergillus* species produced the highest protein yield on the 3rd day when grown in *Aspergillus* Minimal Medium (AMM). In order to assess their effects on *Leishmania* isolates, metabolites from each fungal species were tested at eight different concentrations.

The total protein concentration values of the metabolites produced by *Aspergillus* species in AMM were as follows: sample 2874 ranged from 0.01142105 to 1.4618938 µg/ml, sample 3508 ranged from 0.013 to 1.662 µg/ml, sample 3616 ranged from 0.020 to 2.606 µg/ml, sample 3766 ranged from 0.012 to 1.572 µg/ml, and sample Uşak6 ranged from 0.016 to 2.046 µg/ml when tested on *Leishmania* isolates. BCA assay was conducted, and the *Aspergillus* samples were treated with *Leishmania* isolates. Table 11 presents the BCA assay results for the filtered AMM samples.

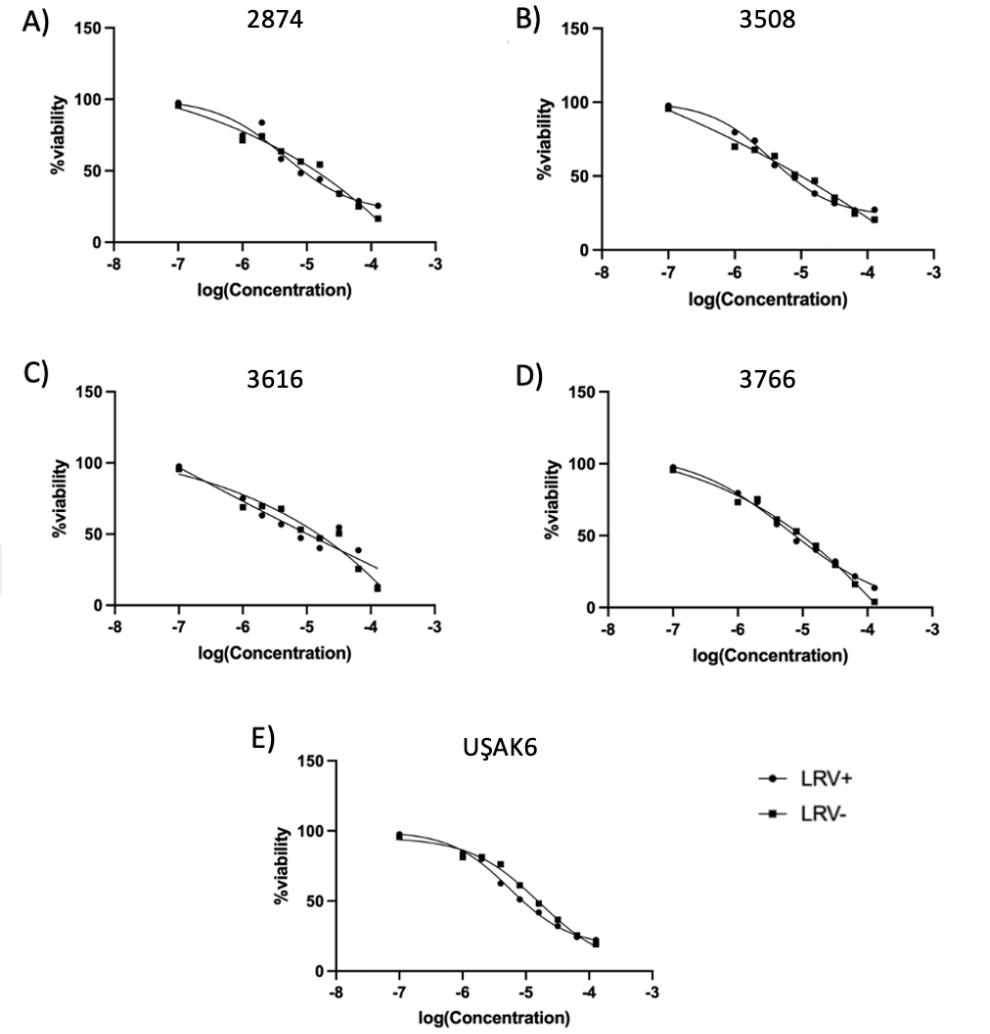

Figure 29. Cell viability of *Leishmania major* LRV+ and LRV- isolates in the presence of metabolites produced by *Aspergillus* species in *Aspergillus* Minimal Medium. These are shown as A) 2874, B) 3508, C) 3616, D) 3766, and E) Uşak6.

Analysis of the XTT assay was shown in Figure 29 and performed using the Excel program. The graphs were created using GraphPad Prism 9 software. Analysis revealed that all metabolites produced in AMM inhibited *Leishmania* isolates. Observing the same pattern across all metabolites led to further investigation to determine whether it was a result of the culture medium itself.

To investigate this, the extent of cell viability variation in *Leishmania* isolates was analyzed in different percentages of Malt Extract Broth (MEB), *Aspergillus* Minimal Medium (AMM), and phosphate-buffered saline (PBS). In the experiment, only

Leishmania isolates grown in 10% FBS-supplemented complete RPMI, without any other medium or buffer, were shown as 0% viability. For example, the 50% sample represents a mixture of 50% RPMI and 50% MEB, AMM, or PBS, as shown in Figure 30.

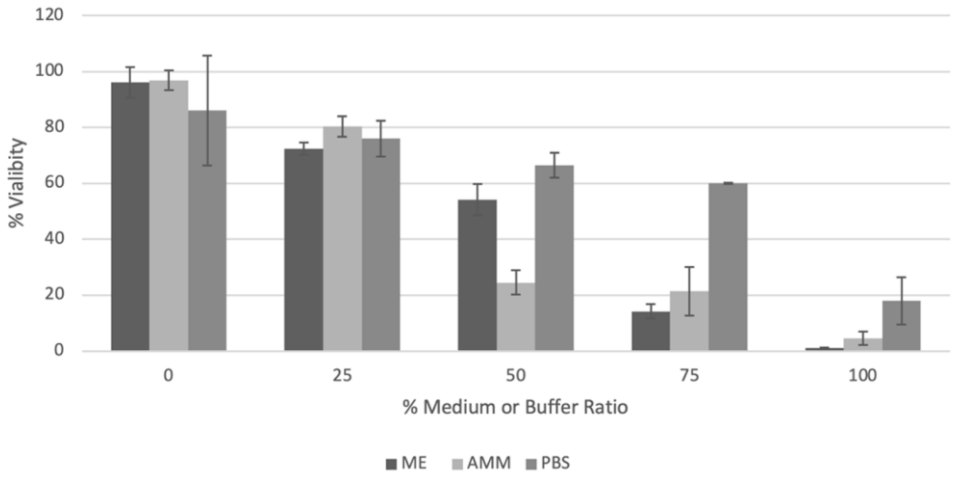

Figure 30. The effect of different media, Malt Extract (ME and Aspergillus Minimal Medium, and PBS buffer on the cell viability of *Leishmania* isolates.

From the results, it can be understood that the columns labeled as 50% represent the highest concentration in the first well, while the 25% column indicates a 1:4 dilution. In the XTT experiments, the second highest concentration of *Aspergillus* samples corresponds to the 25% in this experiment. The remaining six different concentrations of *Aspergillus* samples, which underwent further dilution, exhibit cell viability ranging from 0% to 25%. It can be concluded that the impact of the culture media on *Leishmania* isolates was found to be negligible.

4.6. Molecular Analysis

To verify the functionality of the primers, RNA was isolated and cDNA was synthesized from 1 ml of LRV+ and LRV- cultures that were concentrated to a density of $50x10^6$ cells/ml. The RNA and cDNA samples exhibited the expected purity levels. The concentration of RNA obtained in a volume of 40 ul ranged from 150-200 ng/ul. For cDNA synthesis, an equal amount of RNA was added to each sample, resulting in

the detection of ssDNA with concentrations of 1400-1800 ng/ul using nanodrop measurement.

4.6.1. PCR optimization

After optimizing the conditions for conventional PCR, the primer concentration in the reaction was set to 0.2 mM. In Figure 31, the PCR results for LRV+ and LRV- cell cultures were qualitatively confirmed by comparing them with a 50 bp DNA ladder using 2% agarose gel electrophoresis.

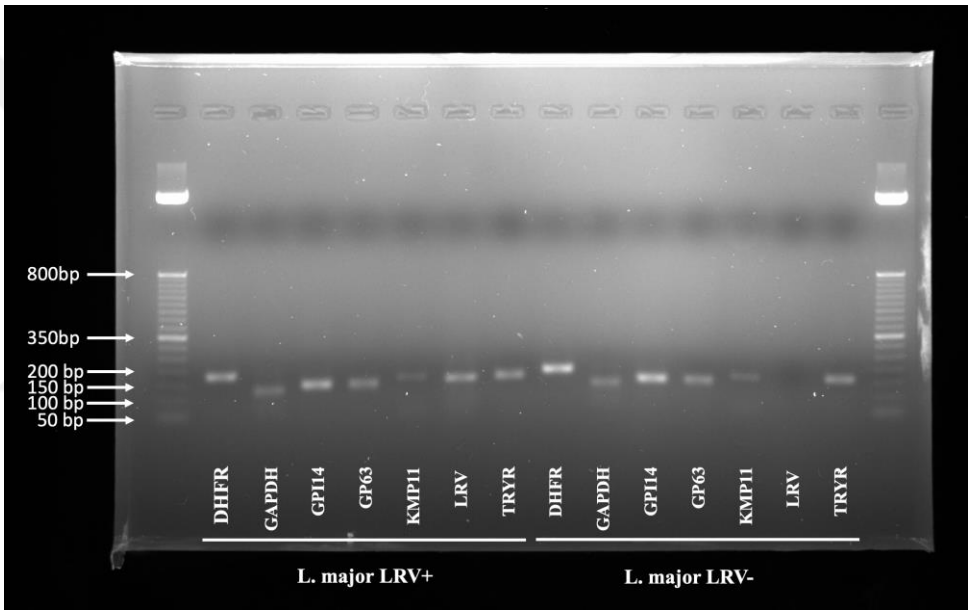

Figure 31. RT-PCR was performed using RNA isolated from Leishmania major LRV+ (left) and LRV- (right) cultures, confirming the functionality of the primers and achieving qualitative differentiation between the two strains. The resulting amplicons appeared as expected, within the anticipated size range of 100-200 bp.

The primer design targeted product sizes of approximately 150-200 bp. The observed product sizes were as follows: DHFR - 178 bp, GAPDH - 123 bp, GPI14 - 142 bp, GP63 - 141 bp, KMP11 - 160 bp, LRV - 155 bp, and TRYR - 155 bp. The gel electrophoresis results shown in Figure 31 are consistent with the expected base pair sizes. LRV- and LRV+ samples were shown using specific primers for Leishmania RNA virus 2 to distinguish between the two samples. The LRV gene was not observed

in the LRV- sample. This qualitative method provided evidence for the first time that the samples were either infected or uninfected with the virus.

4.6.2. Real-Time qPCR

Meglumine antimoniate inhibited L. major LRV+ by 50% at a concentration of 256 μg/ml, while it inhibited LRV- culture by 50% at a concentration of 128 μg/ml. Inspired by these findings, a setup was prepared with concentrations of 128, 256, and 512 μg/ml dissolved in 1% DMSO, with a total of $50x10^6$ cells/ml for each sample. After 72 hours of establishing the experimental setup, the inhibitory values were confirmed by performing an XTT assay on the meglumine antimoniate-treated Leishmania cultures, followed by RNA isolation.

Subsequently, cDNA synthesis was completed, and PCR experiments were performed on GAPDH, KMP11, DHFR, TRYR, and GP63 using the previously optimized PCR conditions. While GPI14 was one of the initially tested primers, due to material limitations, further analysis focused on the genes that showed more significant results.

Initially, GAPDH was selected as the housekeeping gene. However, since the C_T values were not as stable as those of a typical housekeeping gene, KMP11 was included as a second housekeeping gene, which exhibited stability comparable to GAPDH. Therefore, the analysis was conducted with both housekeeping genes as internal controls for comparison. Figure 32 shows the significance of GAPDH/KMP11, which represents the fold change of the GAPDH gene relative to KMP11, and KMP11/GAPDH, which represents the fold change of the KMP11 gene relative to GAPDH. From the results, it can be interpreted that GAPDH is highly expressed in the cells compared to KMP11. Regarding the expression of the GAPDH gene relative to KMP11, the gene regulation in LRV+ initially decreased to 2.7-fold and later reduced to 1.7-fold and 1.2-fold with increasing drug dosage. In contrast, in LRV-, the expression of the GAPDH gene increased up to 2.5-fold with increasing dosage. When interpreting the KMP11 gene relative to GAPDH, in LRV+, at a drug

concentration of 128 µg/ml, it decreases to nearly four-fold, while at 512 µg/ml, it reduces to 1.2 fold. In LRV-, it undergoes downregulation and decreases to 2.5 fold.

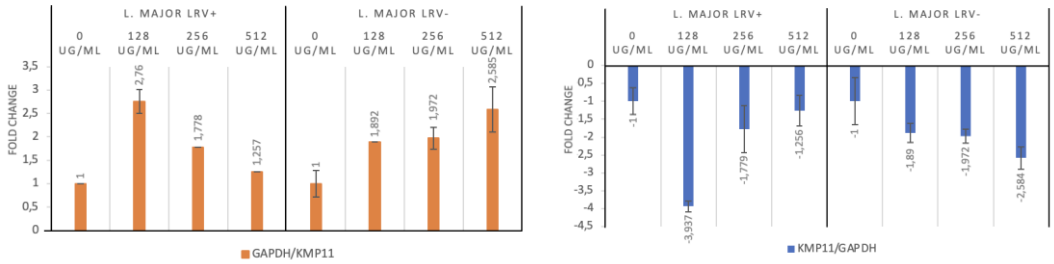

Figure 32. GAPDH/KMP11 and KMP11/GAPDH fold change. The left side represents the interpretation of GAPDH gene expression relative to KMP11, and the right side represents the interpretation of KMP11 gene expression relative to GAPDH. The graphs were generated using Microsoft Excel.

Regardless of whether KMP11 or GAPDH served as internal controls, the expression levels of the DHFR gene increase with increasing drug dosage. The lower C_T values of GAPDH compared to KMP11 indicated differences in fold change within DHFR but consistency within itself. Another point is that higher production occurs in the LRV+ sample compared to LRV- (Figure 33).

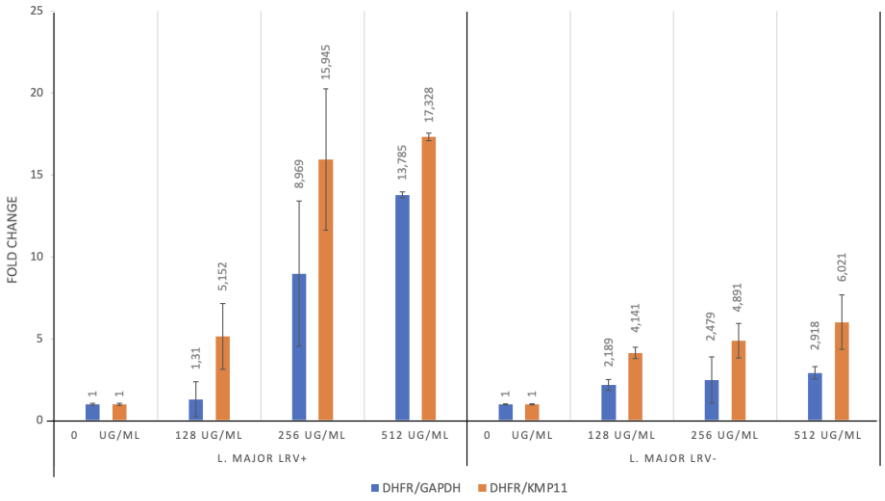

Figure 33. The fold change graph of the DHFR gene relative to GAPDH and KMP11 gene as internal controls. The graph was created using Microsoft Excel.

In the GP63 gene, a general downregulation was observed. Both cultures treated with 256 µg/ml of meglumine antimoniate showed extreme values in fold change. The

LRV- sample exhibited a significantly higher reduction compared to the LRV+ sample. Although GP63 gene expression in LRV+ was affected by the drug, it was not as strongly affected as in the LRV- strain (Figure 34).

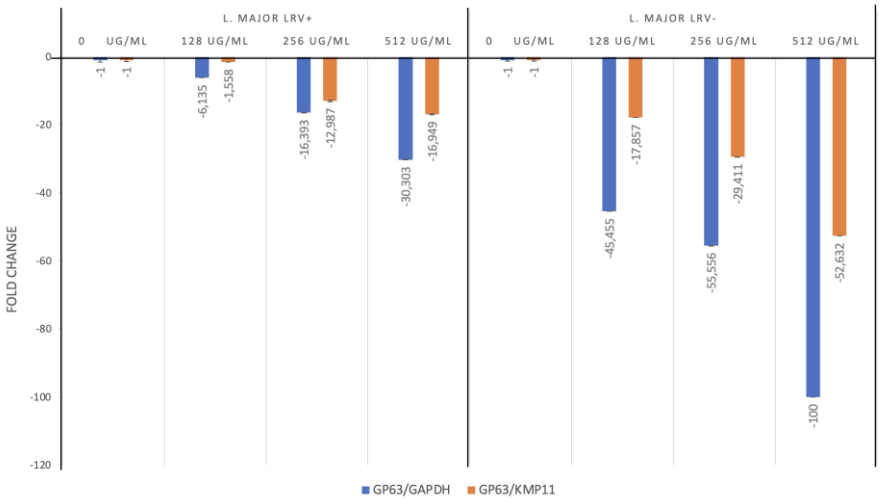

Figure 34. The fold change graph of the GP63 gene relative to GAPDH and KMP11 gene as internal controls. The graph was created using Microsoft Excel.

Lastly, in the TRYR gene, downregulation was observed in Figure 35. In LRV+, with the internal control of GAPDH, a downregulation of 14.925-fold was observed at the 50% inhibitory concentration of 256 µg/ml meglumine antimoniate, while with KMP11, it is 8.474-fold. In LRV-, the 50% inhibitory concentration was 128 µg/ml meglumine antimoniate. With the internal control of GAPDH, it showed a reduction of 16.393-fold, and with KMP11, it was 8.696-fold. The values were found to be close to each other. Except for the 256 µg/ml dosage in LRV-, the expression level of the TRYR gene decreased with increasing drug dosage in all samples.

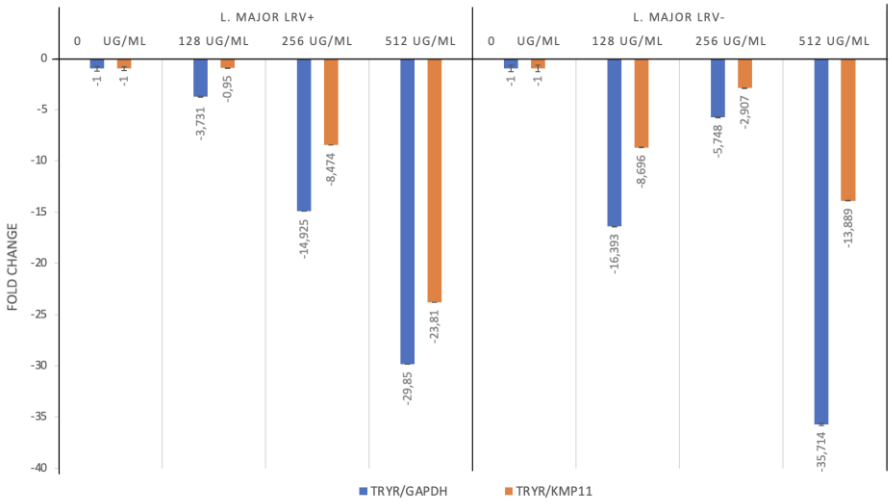

Figure 35. The fold change graph of the TRYR gene relative to GAPDH and KMP11 gene as internal controls. The graph was created using Microsoft Excel.

5. DISCUSSION

During the trials, temperature control was a crucial factor in the reproduction of *Leishmania major* LRV+ and LRV- strains. Even a slight increase of 1-2 degrees in the incubator temperature not only affected the growth of *Leishmania* but also the promastigotes' motility. Observations under the microscope clearly revealed the immobility of the flagella. Once the temperature was controlled, it took 10 days for the LRV+ culture to recover. However, the LRV- isolate was not as adaptable as the LRV+ strain, leading to the loss of the cell line. A new cell line was established, and the experiments continued. Therefore, optimization experiments were conducted using the LRV+ strain, which exhibited an optimized growth curve.

L. braziliensis promastigotes were subjected to temperature effects, and growth curves were examined between 24-30 °C. Although samples at 26 °C appeared to be the most optimal, none of them exhibited a standard growth curve graph. The temperature needed to be optimized for each *Leishmania* species. It can be concluded that the growth curve can be easily influenced by temperature variations (95).

When the growth curves of *Leishmania* isolates were compared, it was observed that L. major LRV+ cells divided more than LRV- cells when cultured at the same concentration. According to Weeks et al.'s study, the higher parasite density in Leishmania cultures with dsRNA is attributed to a decrease in replicase activity. It is believed that there was a linear correlation between cell division and viral replication (96). In a study, it was found that by performing repeated subculturing to maintain a population density between 1×10^6 and 1×10^7 cells ml during the logarithmic growth phase, the doubling time was determined to be 7.1 hours (97).

It is known that fungal spores are resilient and form ubiquitous propagation structures. Spores of Aspergillus strains have been preserved at -80 °C in the research laboratory of Acibadem University for years, and it is also known that they can survive for thousands of years when frozen in soil (Kochkina et al., 2012). Due to the durability and ease of dissemination of the samples, all fungal studies were conducted with great

care. Spores enter the germination stage under suitable conditions. The removal of spores from storage initiates the vegetative growth of a biological organism, followed by both sexual and asexual reproduction for proliferation. However, the signals involved in regulating germination in fungi are poorly defined (98). Similar limitations were encountered in TUCIM samples due to similar reasons. It was not fully understood when *Aspergillus* species grown in dark at 25°C would sporulate. While one of the samples sporulated simultaneously, no sporulation but only the mycelium formation was observed in the other. This problem is universal and is consistent with the literature; the solution lies in optimization experiments that will be conducted over a longer period.

Aspergillus species are known to have good tolerance to temperature and pH. Growth optimization has been achieved at 28°C for eight species, 21°C for seven species, and 14°C for nine species (99). Since the exact identity of the *Aspergillus* strains is unknown, it was observed that they grew sufficiently to cover the plate within 7-8 days at 25°C (Figure 14 and Figure 15).

Aspergillus spp. grown on malt extract agar were collected using Tween80. The dissolution of hyphae coated with hydrophobins in water was quite challenging. Tween80, being a detergent, is useful for dissolving hydrophobic substances. Care was given to collect the spores while excluding mycelial structures. In this stage, the use of miracloth is mentioned in the literature protocols to separate mycelium from the spore solution (100). During the optimization phase, an experiment without miracloth was successful, eliminating the need for miracloth usage.

Observations of *Aspergillus spp.* grown on malt extract agar (MEA) are as follows: The sporangium structures of strain 2874 are very small and highly prone to spreading. Even in a completely closed and motionless state, sporulation was observed to initiate from the center and spread to the periphery (Figure 14). In other samples, growth predominantly exhibited stable expansion around their own surroundings.

The hydrophobin Class I peptides present in *Aspergillus* species can be dissolved in trifluoroacetic acid (TFA) (77). It took approximately 4-5 days for the TFA solution to completely evaporate. Under nitrogen gas, TFA evaporation can be achieved in a much shorter period of time (101).

In liquid cultures of *Aspergillus* species, the presence of hydrophobin structures leads to the formation of mycelial clumps. A study published by Burkholder and Sinnott in 1945 demonstrated the presence of mycelial structures in submerged shaken cultures of fungi (Figure 36) (102). The *Aspergillus* samples used in the study typically exhibited morphological features such as white color and a size of 3-4 mm. Some samples appeared brown due to adhered sporangia, and microscopic examination revealed that the brown portions were conidiophores producing mitotic spores (Figure 19).

Figure 36. Morphological structures formed by different *Aspergillus* species in yeast extract dextrose medium under shaker conditions. Created in biorender.com and taken photograph from Ref (102).

One of the differences observed between malt extract and minimal medium in the study was the formation of mycelial clumps in malt extract, whereas *Aspergillus* minimal medium (AMM) exhibited visible brown aggregated mycelial structures. Despite attempts to remove microorganisms through centrifugation, successful elimination was not achieved in AMM. Sporangia and mycelia did not form structures that could settle in the centrifuge within the culture, resulting in the spores remaining in the supernatant. Before proceeding to XTT assay, the spores were passed through a 0.2 mm sterile filter to purify them. The structures formed in malt extract indicate an abundance of hydrophobic structures, such as hydrophobins. In future studies,

hydrophobic structures can be detected, and different phases can be tested on *Leishmania* isolates through ultrafiltration.

The essential ingredients required for fungal growth in the culture medium are a high carbohydrate source and a nitrogen source. The ideal pH range is between 5 and 6, and the temperature range is 15-37°C (103). The malt extract agar used in the study contains 17.0 g malt extract (carbohydrate source) and 3.0 g mycological peptone (nitrogen source) per liter. The average pH value is 5.4 (104). Malt extract is a rich nutrient source for *Aspergillus* species. In *Aspergillus* minimal medium, there is no nitrogen source, and it only contains 10 g dextrose (carbohydrate source) per liter. This medium serves as the basal medium. The aim here is to investigate the metabolic differences produced by *Aspergillus* species in a rich versus basal medium, specifically focusing on the conditions under which they generate anti-parasitic agents.

In the optimization experiments with *Leishmania* LRV+, a cell count of $10*10^6$ was determined. Additionally, a study was conducted with a positive control using meglumine antimoniate. The cell viability of the LRV+ isolate was found to be approximately 53% at a concentration of 256 μg/ml meglumine antimoniate. Previous studies reported a 50% cell viability for the LRV- isolate at a concentration of 128 μg/ml.

The focus of the study was on the protein metabolites produced by *Aspergillus* samples in different media. The BCA assay was used to determine the total protein concentration (Figure 22 and Figure 23). When protein concentrations were not estimated, dilutions were performed, and optimization was carried out with individual samples. Initially, the protein concentrations of the metabolites produced in malt extract ranged from 2.5 to 3.5 mg/ml, while in the basal medium, they were calculated to be in the range of 1.2 to 1.8 mg/ml. The variation in protein levels in the environment was confirmed. Protein levels increased as the days progressed in sample 3616, while in other samples, they decreased until the third day, reached their highest values on the fourth day, and then decreased again. The highest protein level, exceeding 1.8 mg/ml, was calculated in sample 3616. When the metabolites obtained from AMM were tested

on *Leishmania* isolates, results were obtained that affected the growth of *Leishmania* in all samples. According to the results, the metabolites produced by *Aspergillus* species under stress conditions inhibit the activation of *Leishmania*. When looking at the IC50 values of *Aspergillus* samples, samples 2874 and 3508 inhibited at a concentration of 0.1 mg/ml, while Uşak6 inhibited below 0.15 mg/ml, and samples 3766 and 3616 were around 0.2 mg/ml. Although sample 3616 had a higher protein concentration, samples 2874 and 3508 were more effective. The experiments conducted here were to determine the initial potential. The next step is to detect the protein through SDS-PAGE and identify the active substance through LC/MS in further studies.

The total protein amounts and pH values of *Aspergillus* samples grown in malt extract medium are presented in Figure 23. The most notable observation here was the low pH values. This is due to the production of citric acid by *Aspergillus*. Especially *A. niger* is widely used as the main organism for fungal fermentation in commercial citric acid production. In fungal cultures, pellet form is desired for citric acid production (105). When looking at the samples, the most acidic environment belongs to sample 2874, and it can be assumed that this sample produces the most citric acid. In terms of total protein content, sample 2874 has approximately 0.5 mg/ml higher values compared to the others. The highest protein production for all samples occurred on the fourth day before the XTT assay. The subsequent decrease in protein concentration may be due to the utilization of the produced proteins for secondary metabolite production. When examining the total protein values on the fourth day, samples 2874 and 3508 produced over 2.5 mg/ml. While sample 3616 was above 2 mg/ml, samples 3766 and Uşak6 showed protein levels below 2 mg/ml. Since the pH values of the samples were below 7, they were adjusted using KOH. KOH is also used for the neutralization of citric acid (106). BCA assay was performed again after dilution, and the results are shown in Table 10. In terms of their effects on Leishmania isolates, it can be observed that samples 2874, 3508, and 3766 have anti-leishmanial activity. Here, LRV+ isolates are more sensitive compared to LRV- isolates. The best results are obtained in sample 3766, with IC50 values between 30-70 μg/ml for both LRV+ and LRV-. The IC50 value for sample 2874 is around 300 μg/ml. Since samples

2874 and 3508 achieved inhibitory effects at the last two concentrations, future studies should include higher concentration samples as well.

Regarding the samples obtained from *Aspergillus* Hydrophobin Class I, samples 2874 and 3766 are effective for both LRV+ and LRV- isolates. The initial potential of sample 3766 has been observed, and in future studies, it should be tested with higher concentrations. Samples 3616 and Uşak6 were effective for LRV+ but not for LRV- isolates. Sample 3508 did not show any anti-leishmanial effect. TFA-treated samples were more successful in LRV+ compared to LRV-. In this case, besides antileishmanial activity, an antiviral agent may have been obtained. In further studies, the active substance should be identified using the previously mentioned methods, and the study should proceed by fractionating the samples.

In future studies, it is planned to separate the metabolite samples into different phases using ultrafiltration and conduct experiments. Additionally, methods such as SDS-PAGE and LC/MS should be used to identify the active substance. The obtained results have identified the initial potential samples and aimed to advance the study through the implementation of cytotoxicity assays, determination of the active substance, and further investigations, including higher concentration samples.

In this study, the aim was to investigate the effects of effective fungal samples and the positive control, meglumine antimoniate, on gene groups in specific pathways in *Leishmania major* LRV+ and LRV- samples. Comparing the highly specific *L. major* LRV+ and LRV- samples with the positive control provides unique insights. Therefore, further studies will be conducted on the effective fungal samples.

To optimize PCR, DNA isolation was performed. The proteinase K step was extended during the extraction of Leishmania, and physical disruption was necessary to obtain high-quality DNA. Zirconia beads were preferred for this purpose, resulting in improved DNA quality. The DNA quantity, primer concentration, and PCR conditions were optimized for conventional PCR. The PCR products were analyzed by electrophoresis on a 2% agarose gel. During gel electrophoresis, it is important to

maintain a pH of 8.3 for the Tris-Acetate-EDTA (TAE) Buffer to ensure proper electrophoretic migration. Primer-dimer formation and the size of PCR products were evaluated on the gel (Figure 31). Additionally, specific primers targeting the capsid protein of LRV were used to differentiate between *L. major* LRV+ and LRV- samples.

The cell number to be used for RNA isolation was optimized to 50 x 10^6 cells. A larger scale was required for the experimental setup previously planned for XTT assay. Due to the slow growth rate of *Leishmania*, only different doses of meglumine antimoniate could be studied. RNA isolation and cDNA synthesis steps were optimized.

The first step in RNA isolation was adding beta-mercaptoethanol (ß-ME) to the culture. This is because intracellular RNases are released during the lysis step of the RNA isolation procedure, and they need to be quickly and completely inactivated to obtain high-quality RNA. ß-ME is a reducing agent that irreversibly denatures RNases by reducing disulfide bonds and disrupting necessary natural conformations for enzyme functionality (107).

For the qPCR stage, Glycerol-3-phosphate dehydrogenase (GAPDH) was chosen as the internal control gene. Although GAPDH is the most well-known and commonly used internal control, drugs that affect GAPDH gene expression have been identified in *Leishmania* (54).Since there is no literature indicating changes in GAPDH with meglumine antimoniate, it was used as the internal control for qPCR. Additionally, another internal control, Kinetoplastid membrane protein-11 (KMP11), which maintains stability similar to GAPDH, was calculated.

KMP11 was initially identified in *Leishmania donovani* associated with the lipophosphoglycan (LPG) molecule and is found throughout the parasite surface. The 11 kDa molecule was first isolated from *Leishmania donovani*, and its primary structure was determined by protein and DNA sequencing (Jardim et al., 1995). KMP11 protein is primarily localized around the flagellum and flagellar pocket. It has been observed that KMP11 is induced by pentamidine and sulforaphane (108).

The qPCR results were analyzed using Ct values, and the melt curve profiles were checked for quality control. The Ct values were calculated using the $2^{-\Delta\Delta CT}$ equation. For genes showing downregulation, fold change was calculated as -1/2^(-ΔΔCT). All genes studied in this work were presented in a comparative manner under two conditions where GAPDH and KMP11 were considered as internal controls. The varying gene expression levels were examined in *L. major* LRV+ and LRV- cultures treated with meglumine antimoniate at concentrations of 128, 256, and 512 µg/ml. In the experiments, the 128 µg/ml drug dose inhibited the LRV- sample by 50%, while the 256 µg/ml drug dose inhibited the LRV+ sample.

Meglumine antimoniate (Glucantime) is a pentavalent antimony compound (SbV) recommended as the first-line drug by the World Health Organization for the treatment of all types of leishmaniasis. Despite being used for over 60 years, the pharmacological and toxicological mechanisms involved in their effects remain unclear (109). It has been proposed that pentavalent antimonials such as SbV interfere with the bioenergetic processes of *Leishmania* amastigotes by forming stable complexes with ribonucleosides, thereby disrupting the parasite's fatty acid β-oxidation and glycolysis, leading to depletion of intracellular ATP levels (28,30). Another hypothesis suggests the conversion of the drug into a more toxic trivalent form (SbIII), which exhibits anti-leishmanial activity (110). Additionally, it has been demonstrated that meglumine antimoniate can cause DNA damage in vivo, not just in vitro (109). SbIII has been observed to alter intracellular thiol flux and inhibit trypanothione reductase, thereby affecting cellular redox potential (22,24). Moreover, studies have shown that antimonial compounds can modify enzyme activities associated with the antioxidant defense system, leading to increased levels of reactive oxygen species (ROS)/reactive nitrogen species (RNS) (111). Consequently, meglumine antimoniate can exist in two forms, Sb (V) and Sb (III), and has been shown to induce DNA damage in vivo, in accordance with in vitro findings. It is generally known to induce oxidative stress. The experimental results will be interpreted based on this information.

The KMP11 gene is expressed at lower levels in LRV+ and LRV- strains compared to the control group, whereas the GAPDH gene appears to be more highly

expressed than the control group relative to KMP11. Since this is a relative evaluation, it is not considered an absolute truth but rather a comparative analysis. In LRV+, KMP11 expression increases with drug dosage relative to GAPDH, while in LRV-, it decreases.

The GP63 gene is one of the surface proteins considered potential virulence factors that enable *Leishmania* to evade the host macrophage defense system and, therefore, survive. GP63 was initially discovered in the 1980s and defined as a major surface antigen expressed in *Leishmania* promastigotes from various species (Fong and Chang, 1982; Bouvier et al., 1985; Etges et al., 1985; Chang et al., 1986). Initially, it was identified as having protease activity and was called the major surface protease (MSP), but later it was characterized as a zinc metalloprotease (Etges et al., 1986; Chaudhuri and Chang, 1988). Due to this property, GP63 was named leishmanolysin by the International Union of Biochemistry and Molecular Biology (IUBMB) Enzyme Nomenclature. Metalloproteases like GP63 are not only present in different *Leishmania* species but also in various Trypanosoma species and *Trichomonas vaginalis* (112).

In the studies conducted on Leishmania RNA Virus 2, *L. major* LRV+ and LRV- promastigotes were tested on THP-1, a human leukemia monocytic cell line, and the gene expression levels of Glycoprotein 63 (GP63) and several other gene groups were examined using real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The findings revealed an increase in GP63 gene expression in the presence of LRV+ (50). GP63 gene is known as a virulence factor that acts against the macrophage defense system, therefore an increase in its expression was expected in the THP-1 cell line. However, this could be different in the parasite culture environment, and this thesis focuses on the GP63 gene expression levels of promastigotes in an in vitro culture environment.

When examining the expression levels of the GP63 gene, down-regulation was observed in all samples. However, in the *L. major* LRV- sample, the fold change was higher. Based on the interpretation relative to GAPDH, at a drug dosage of 512 ug/ml,

the GP63 gene decreased by 16-fold in LRV+ while increasing by 45-fold in LRV-. There was nearly a 3-fold difference. Considering that GP63 was a surface-active protein that could affect a virulence factor, the LRV+ sample was less affected compared to LRV-, indicating that LRV+ was more adaptive and had higher virulence.

Dihydrofolate reductase (DHFR) catalyzes the reduction of dihydrofolate to tetrahydrofolate (THF). THF is necessary for the functioning of folate-dependent enzymes and is therefore essential for DNA synthesis and methylation (113). The protozoan parasite Leishmania is auxotrophic for folates and unconjugated pterins, both of which it salvages from mammalian hosts. Two enzymes in the folate metabolism pathway, bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) and pteridine reductase 1 (PTR1), play crucial roles in this process (47). From a medicinal chemistry perspective, DHFR is particularly interesting because it is an ubiquitous enzyme involved in folate metabolism and required for cell proliferation and purine and thymidylate synthesis. Impaired DHFR activity leads to tetrahydrofolate deficiency and cell death (114).

In this thesis, due to the importance of the folate metabolism pathway, the DHFR gene was investigated in an in vitro assay, with GAPDH and KMP11 serving as internal controls. A minimum 2-fold increase in DHFR gene expression was observed. The increase in DHFR expression is higher in LRV+. Since the internal controls are consistent with each other, an up-regulation is observed for DHFR in this case. The reason for a higher fold increase in LRV+ is not yet known in the literature. It could be induced by another gene pathway or the presence of a virus.

Parasitic protozoa belonging to the Kinetoplastida order contain trypanothione as their main thiol. Tripanothione reductase (referred to as TRYR or TR) is the enzyme responsible for maintaining the reduced form of trypanothione and is believed to be at the center of the redox defense systems of trypanosomatids (115). It is known that meglumine antimoniate in its Sb(III) form causes inhibition of trypanothione reductase (22,24).

The effectiveness of drugs varies between the amastigote and promastigote forms of *Leishmania*. A research group investigated the efficacy using a TRYR-based assay. They examined the IC50 values of amphotericin B, miltefosine, pentamidine, and paromomycin in *L. major* and *L. donovani* strains. In *L. major*, amphotericin was three times more effective in the promastigote form compared to the amastigote form, while miltefosine was six times more effective in the amastigote form compared to the promastigote form. Thus, it has been demonstrated that the efficacy of drugs varies depending on the form of *Leishmania* (116).

The TRYR gene, considered as one of the key genes in the mechanism of action of meglumine antimonate, is also known to be affected by the development of resistance to the drug. For example, in a study conducted on 15 clinical resistant *L. tropica* strains, structural mutations observed in 9 amino acids surrounding the active site of the TRYR gene altered the three-dimensional structure of trypanothione reductase. These findings suggest a potential association between these mutations and resistance to meglumine antimonate (117). *Leishmania* parasites gradually develop resistance to meglumine antimonate over time, but the effectiveness of this mechanism in the presence of LRV has not yet been investigated.

As seen in Figure 35, the trypanothione reductase gene showed a proportional down-regulation in response to meglumine antimoniate. Compared to the GAPDH internal control, at the 50% inhibitory dose of 128 ug/ml, *L. major* LRV- exhibited a 16-fold decrease, while LRV+ showed a 15-fold decrease at the 50% inhibitory dose of 256 ug/ml. Similar values were obtained at the lethal dose, indicating a direct association between the drug and the TRYR gene. The findings support the hypothesis of the conversion of meglumine antimoniate, a pentavalent antimony compound, from Sb(V) to Sb(III) form.

In future studies, along with testing pre-designed primers for all affected vital pathways under in vitro conditions to determine which pathway is involved, the aim is to advance the research to the in vivo stage, contributing to the elucidation of the mechanism of action of the compound meglumine antimoniate. Additionally,

molecular-level analysis of the *Aspergillus* samples, completion of cytotoxicity studies, and initiation of advanced studies with suitable samples for in vivo experiments are planned.

6. CONCLUSION

The aim of this study was to detect the differences in gene expression levels between *Leishmania major* isolates with *Leishmania* RNA Virus (LRV+) and without the virus (LRV-) at the 50% inhibitory dose of meglumine antimoniate through molecular analysis. Additionally, the study aimed to investigate the anti-leishmanial activity of small cysteine-rich peptides (Hydrophobin Class I) and metabolites produced by five different Aspergillus species (TUCIM 2874, 3508, 3616, 3766, and Uşak6) on *L. major* LRV+ and LRV. The research involved the cultivation of *Leishmania* isolates and Aspergillus spp. cultures, extraction of hydrophobins obtained through treatment with TFA, and the isolation of metabolites produced by *Aspergillus* in rich and basal metabolism media. Total protein concentration was determined using the BCA assay, and cell viability studies were conducted using the XTT assay to identify strains with initial potential agents.

The *L. major* LRV+ isolate was inhibited by meglumine antimoniate at 256 ug/ml, while the LRV- isolates exhibited inhibition at 128 ug/ml. Cultures were treated with 128 ug/ml, 256 ug/ml, and 256 ug/ml meglumine antimoniate on a large scale, and after 72 hours, RNA was obtained and cDNA was synthesized for Real-time PCR analysis of the DHFR, GP63, and TRYR genes. GAPDH was used as an internal control, and the KMP11 gene was used as a second control. The DHFR gene showed increased up-regulation with the drug dose in both isolates, although it was higher in the LRV+ isolate. While both isolates exhibited down-regulation in the GP63 gene, the LRV- isolate showed a threefold higher down-regulation. The TRYR gene, known to be inhibited by Sb(III) antimony compounds in the literature, exhibited down-regulation proportional to the drug dose, with higher down-regulation in LRV+. Similar results were obtained for GP63 and TRYR genes as reported in the literature. However, the literature regarding DHFR gene in vitro studies is uncertain.

Aspergillus spp. is commonly used as a host organism for enzyme production in industries. While there are only a few research groups studying the anti-leishmanial activity of *Aspergillus*, the fact that the widely used leishmaniasis drug, amphotericin

B, has a fungal origin, the structural similarities between fungi and protozoan parasites, and the similarity in the mechanisms of action and functions of antimicrobial peptides with leishmaniasis drugs indicate the need for further studies in this field. Additionally, there is a lack of studies on the interaction between fungi and *Leishmania major* strains with and without viruses. The results obtained from the studies showed variations in the responses of LRV+ and LRV- isolates to fungal samples. For example, the action potential of hydrophobin class I peptides was higher on LRV+ isolates compared to LRV-, while the positive control, meglumine antimoniate, showed the opposite pattern, being more inhibitory on LRV+ isolates compared to LRV-.

As *Aspergillus* minimal medium has low nutritional value, *Aspergillus spp*. underwent stress and produced different metabolites. The metabolites produced in all samples showed an IC50 value of approximately 100 μg/ml for both LRV+ and LRV- isolates. In malt extract medium, citric acid production was observed, and the pH values were maintained at a low level. Effective anti-leishmanial activity was observed in *Aspergillus* samples 3508, 2874, and 3766, which were obtained from malt extract. Samples 2874 and 3508 showed efficacy against Leishmania with all three methods employed to obtain *Aspergillus samples.*

In conclusion, this study investigated the mechanisms of action of meglumine antimoniate in the presence of *L. major* LRV through molecular analysis. Future studies aim to expand the investigation of the effects on other vital pathways and explore the mechanism of action of the drug in vivo. The study also demonstrated the potential leishmanial effects of *Aspergillus* hydrophobin Class I peptides and metabolites produced under different conditions. This thesis represents the first step in this research field, and future studies are planned for the identification of active compounds through cytotoxicity testing and for more comprehensive investigations.

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8. APPENDIX

Appendix 1 Growth belongs to TUCIM number 3508 and 3766 from *Aspergillus* species on malt extract agar was respectively shown in Figure 37 and Figure 38.

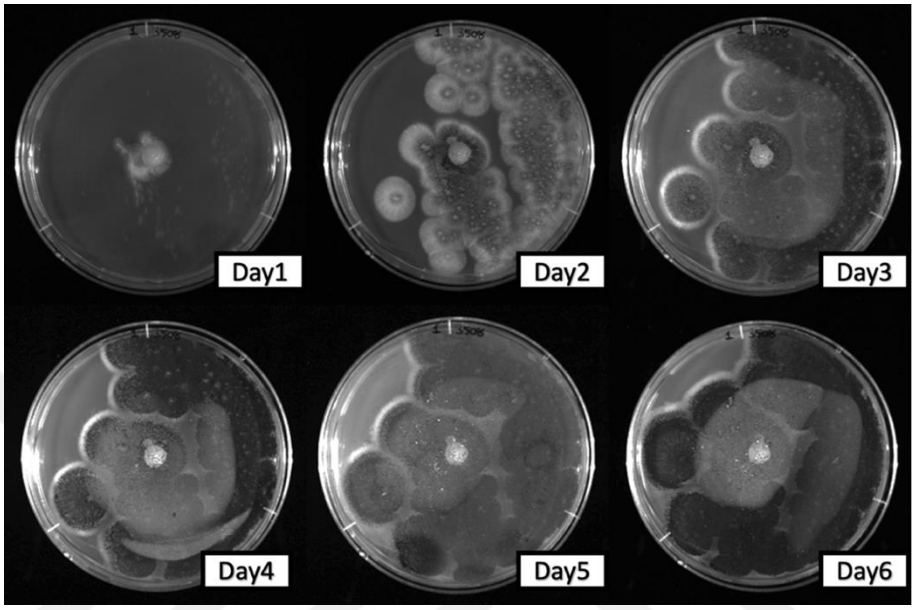

Figure 37. The daily observation of sample 3508 from *Aspergillus* species on malt extract agar.

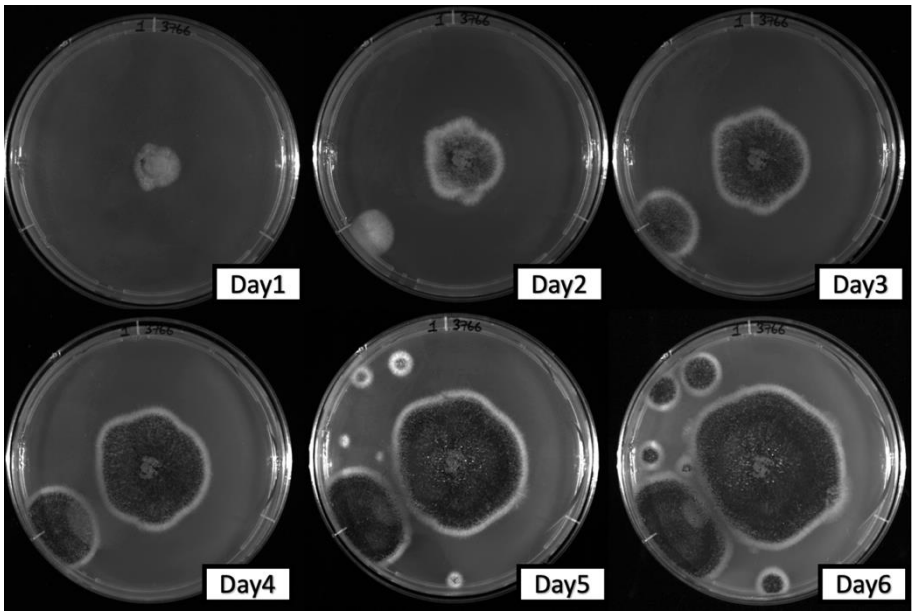

Figure 38. The daily observation of sample 3766 from *Aspergillus* species on malt extract agar.

Appendix 1 (Cont'd) Growth belongs to TUCIM number UŞAK& from *Aspergillus* species on malt extract agar was shown in Figure 39.

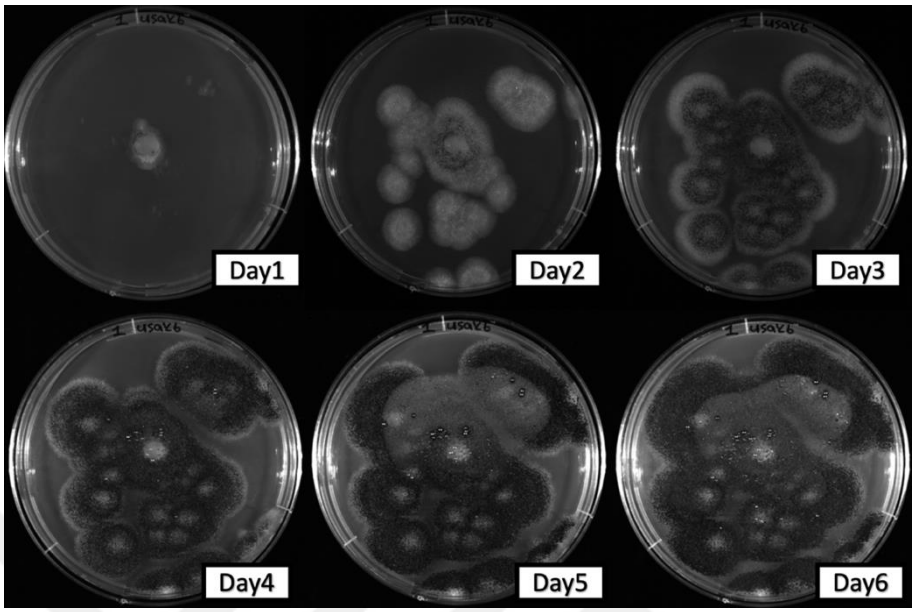

Figure 39. The daily observation of sample UŞAK6 from *Aspergillus* species on malt extract agar.

Appendix 2 The mycelium structure of five different *Aspergillus* strains on malt extract broth under the microscope that shown in Figure 40.

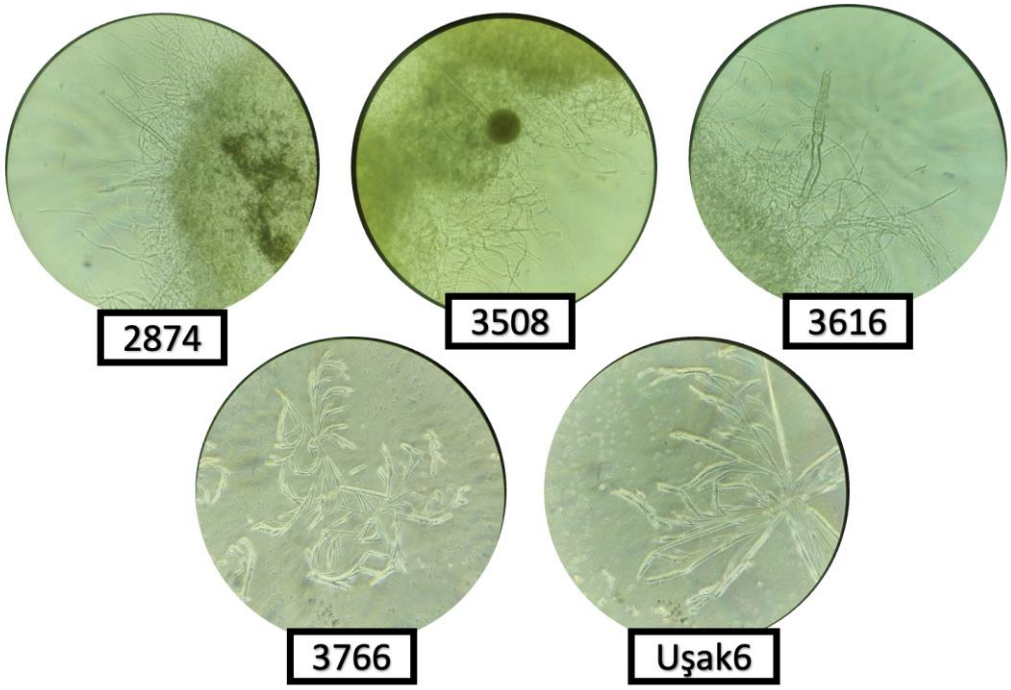

Figure 40. Mycelial and sporangial structures of *Aspergillus* strains grown in Malt Extract Broth.

Appendix 3 The mycelium structure of five different *Aspergillus* strains on *Aspergillus* minimal medium under the microscope that shown in Figure 41.

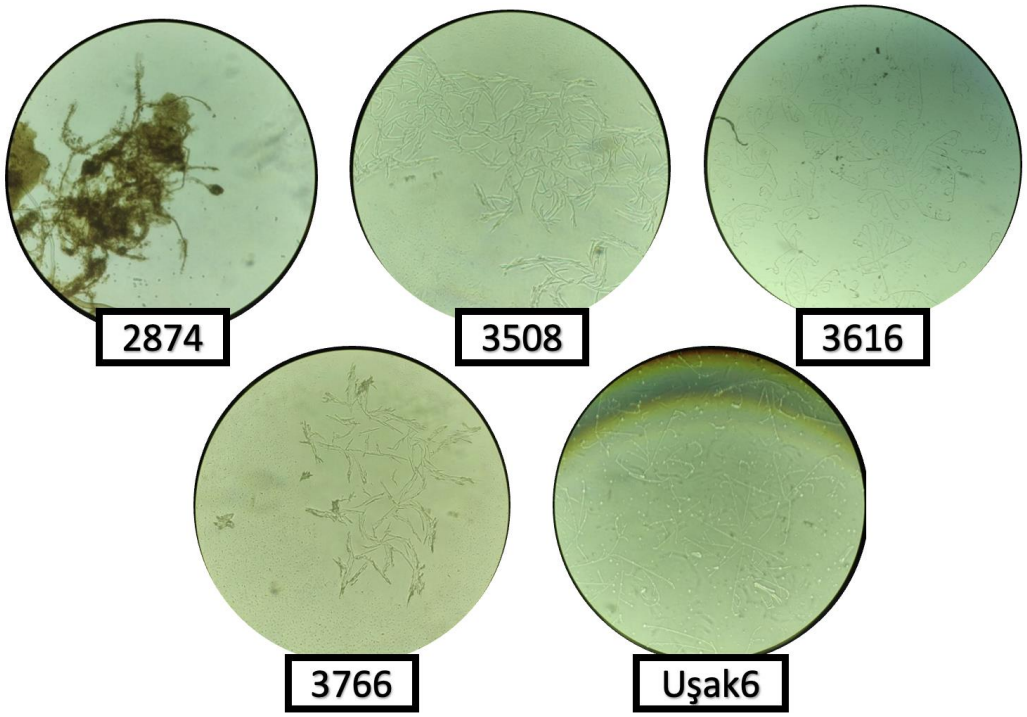

Figure 41. Mycelial and sporangial structures of *Aspergillus* strains grown in *Aspergillus* Minimal Medium

9. CURRICULUM VITAE