Chapter- 1

Introduction

1. Introduction

A multitude of parasites, spanning from protozoa to helminths, have the potential to endanger human health. Parasites responsible for various diseases are characterized by heightened levels of both morbidity and mortality. To show its influence, diverse parasitic species employ a range of infection mechanisms across various host species [1]. This variation contributes to the adaptability and complexity of parasitic infections in different hosts. Certain parasitic organisms which cause diseases like Chagas, Leishmaniasis, Schistosomiasis, etc., fall under the classification of infectious diseases known as Neglected Tropical Diseases (NTDs). The data indicates that NTDs are primarily prevalent in countries across Africa, Asia, and some parts of South America [2].

1.1. Leishmaniasis- An overview

Leishmaniasis is one of the NTDs which affects many countries across the globe and it stands out as a crucial parasitic ailment, contributing substantially to global mortality and morbidity. An intracellular protozoan parasite belonging to the *Leishmania* genus is required to spread this disease through a vector [3]. Leishmaniasis are believed to impact approximately 0.7–1 million individuals, with a global population of 350 million people facing the risk of infection. According to global data provided by the World Health Organization (WHO), there are over 12 million individuals currently infected, with an annual incidence of new cases ranging between 0.9 to 1.6 million. The disease leads to an estimated 20,000 to 30,000 deaths annually, and a staggering 350 million people are at risk of contracting the infection [4]. Moreover, among the 30 identified species of the *Leishmania* parasite to date, 21 have been documented as responsible for causing the disease known as leishmaniasis in humans. These 21 species of *Leishmania* contribute to distinct forms of leishmaniasis, namely cutaneous, mucocutaneous, and visceral leishmaniasis [3].

1.2. Etiology

Leishmaniasis results from the infection caused by the *Leishmania* parasite, with the female sand-fly Phlebotomus acts as the vector [5]. The initiation of leishmaniasis disease occurs when parasites are conveyed to the mammalian host by the means of blood-feeding activity of an infected female sandfly. The parasites, existing in two forms, enter the human host as metacyclic promastigotes, and subsequently undergo a transformation into their immobile amastigote form [6]. The array of *Leishmania* species as mentioned, includes *L. donovani, L. major, L. braziliensis, L. infantum, L. mexicana,* and *L. chagasi* that gives rise to various

forms of Leishmaniasis. The spectrum of disease severity encompasses individuals who are serologically positive but asymptomatic, all these cases marked by disfiguration and the potential for fatal outcomes [7]. Based on the clinical presentation, leishmaniasis is categorized into four primary forms. The most life-threatening form of leishmaniasis, Visceral Leishmaniasis (VL), is caused by *L. donovani* and *L. infantum.* When VL establishes itself in the host, symptoms include the enlargement of the liver and spleen, accompanied by anemia, weight loss, and intermittent fevers [8]. In context of India, individuals affected by VL exhibit skin darkening, a phenomenon attributed to the cytokine-triggered synthesis of adrenocorticotrophic hormone. Thus, VL is considered as "kala-azar" in India [9]. Cutaneous leishmaniasis (CL), a non-fatal variant of leishmaniasis, is prominently caused by numerous *Leishmania* species, including but not limited to *L. major, L. tropica, L. amazonesis, L. braziliensis, L. mexicana*, *L. panamensis*, and others. The lesions occurred by CL are confined to the site and may undergo self-healing [10]. Nevertheless, the visual characteristics and duration of healing differ among species, often resulting in notable scarring and an extended healing period spanning several years. Mucocutaneous leishmaniasis (MCL), a less common form of leishmaniasis, manifests on the mucous membranes of the face, including the nose, mouth, and throat. In this form, parasites can access the mucosa either through the dissemination via the lymphatic system or by direct injection into the region through the bite of an infected sandfly [11]. Manifestations of this leishmaniasis encompass symptoms such as nasal discharge, congestion, and episodes of sudden nosebleeds, which may manifest years following a starting cutaneous lesion [12].

1.3. Epidemiology

The prevalence of leishmaniasis exhibits a broad geographical distribution, with reported cases spanning the globe. Regions characterized by inadequate sanitation and substandard living conditions foster an elevated proliferation of vectors, contributing to a higher incidence of cases, particularly in developing nations [13, 14]. As of November 2023, the WHO released a report detailing the global endemic status of CL of 2022. Currently, approximately 95% of endemic cases of the cutaneous form are concentrated in regions including America, the Mediterranean basin, the Middle East, and countries in central Asia. Some of the countries where the cutaneous form is endemic are Brazil, Colombia, Ethiopia, Sudan, Afghanistan, Iran, Pakistan, Tunisia, etc., [15]. Regarding VL, WHO presented the worldwide endemic situation in 2022. The report highlighted countries like India, South

Sudan, Sudan, Brazil, Ethiopia and Somalia as having the highest incidence of VL cases [15]. The prevalence of VL is disproportionately concentrated in these nations, accounting for 90% of all reported cases. VL is extensively documented in numerous countries, with India and Sudan being a significant contributor to the global burden [16]. India accounts for 18% of the global burden of VL in 2020, emphasizing its substantial impact on the prevalence of the disease [17]. In India, the primary states reporting VL cases are Bihar, Jharkhand, West Bengal and Uttar Pradesh, according to information obtained from the National Vector Borne Disease Control Programme (NVBDCP) of India [18]. MCL has been documented in regions spanning South America, Asia, Europe and Africa. Noteworthy countries reporting MCL cases include India, Sri Lanka, Pakistan, Iran, Saudi Arabia and Sudan [19]. WHO presented the global endemic situation of CL and VL in 2022 through Figures 1.1 and 1.2. Additionally, Figure 1.3 depicted the distribution of different species of Leishmaniasis in Indian subcontinent.

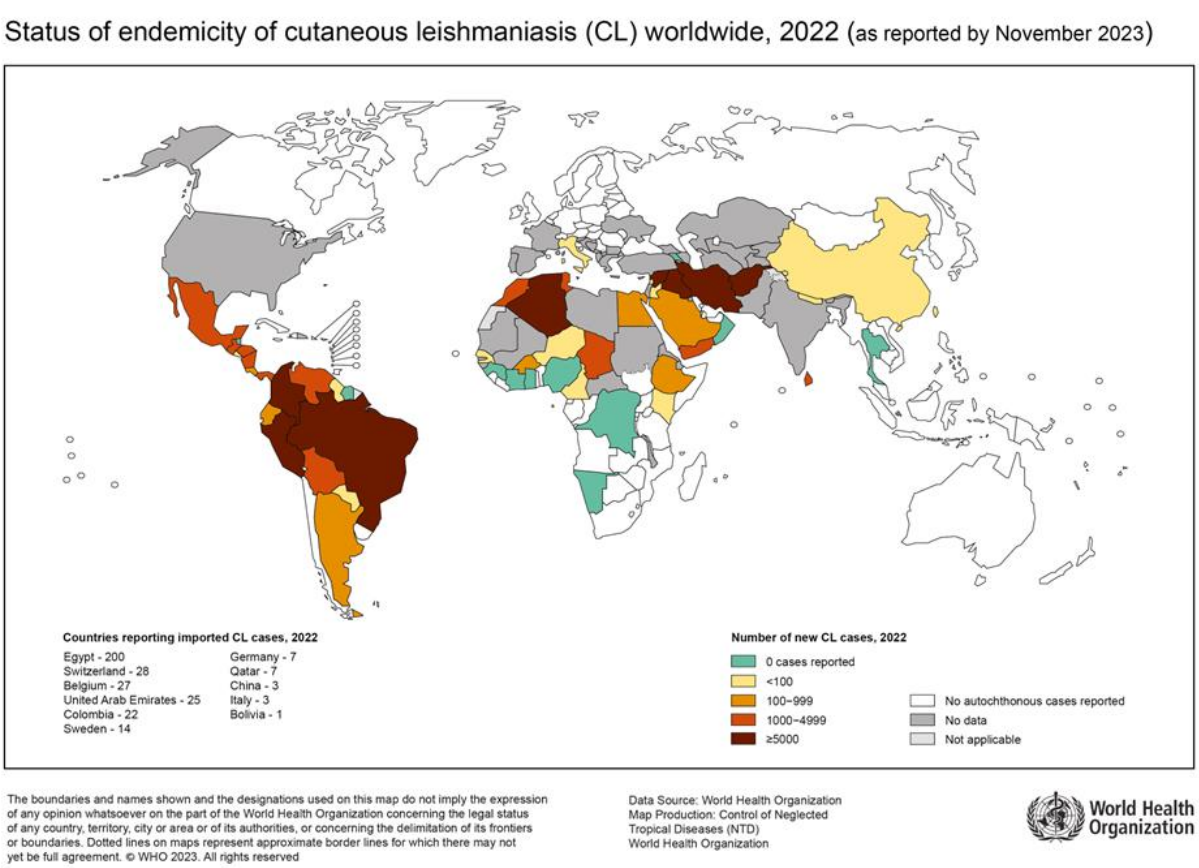

Figure 1.1: Endemic status of CL 2022 taken from WHO

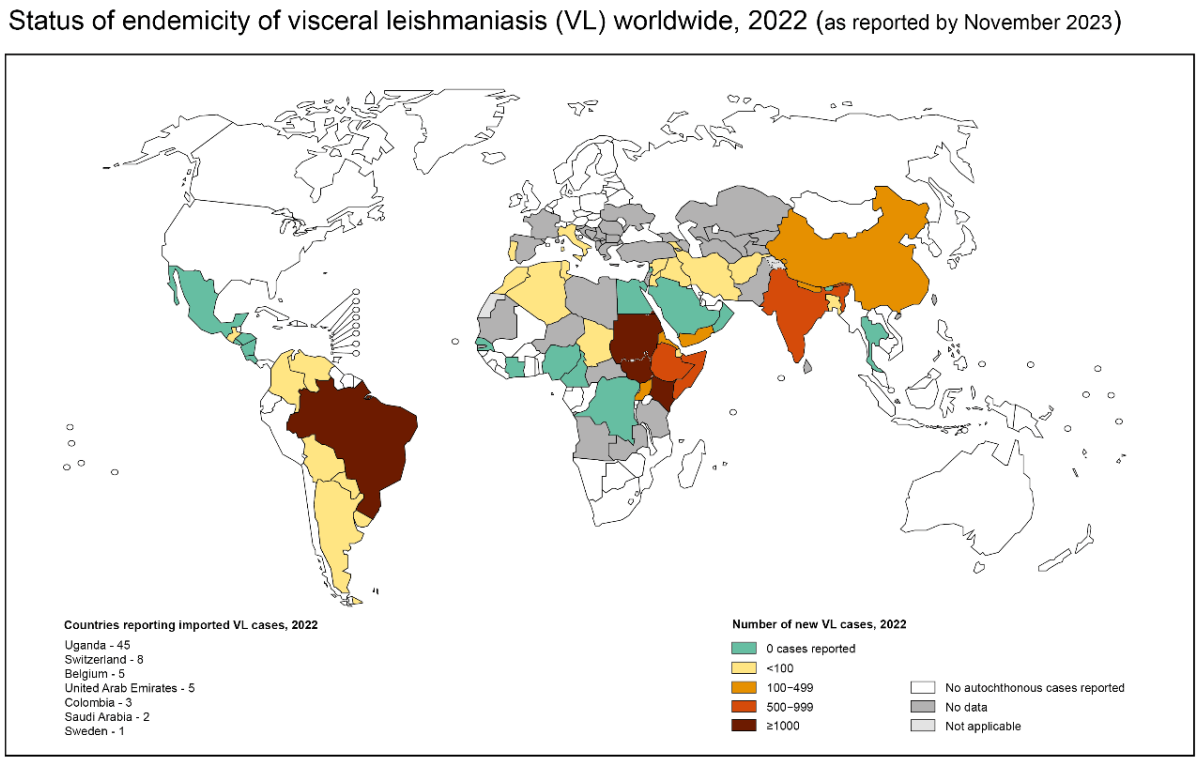

Figure 1.2: Endemic status of VL 2022 taken from WHO

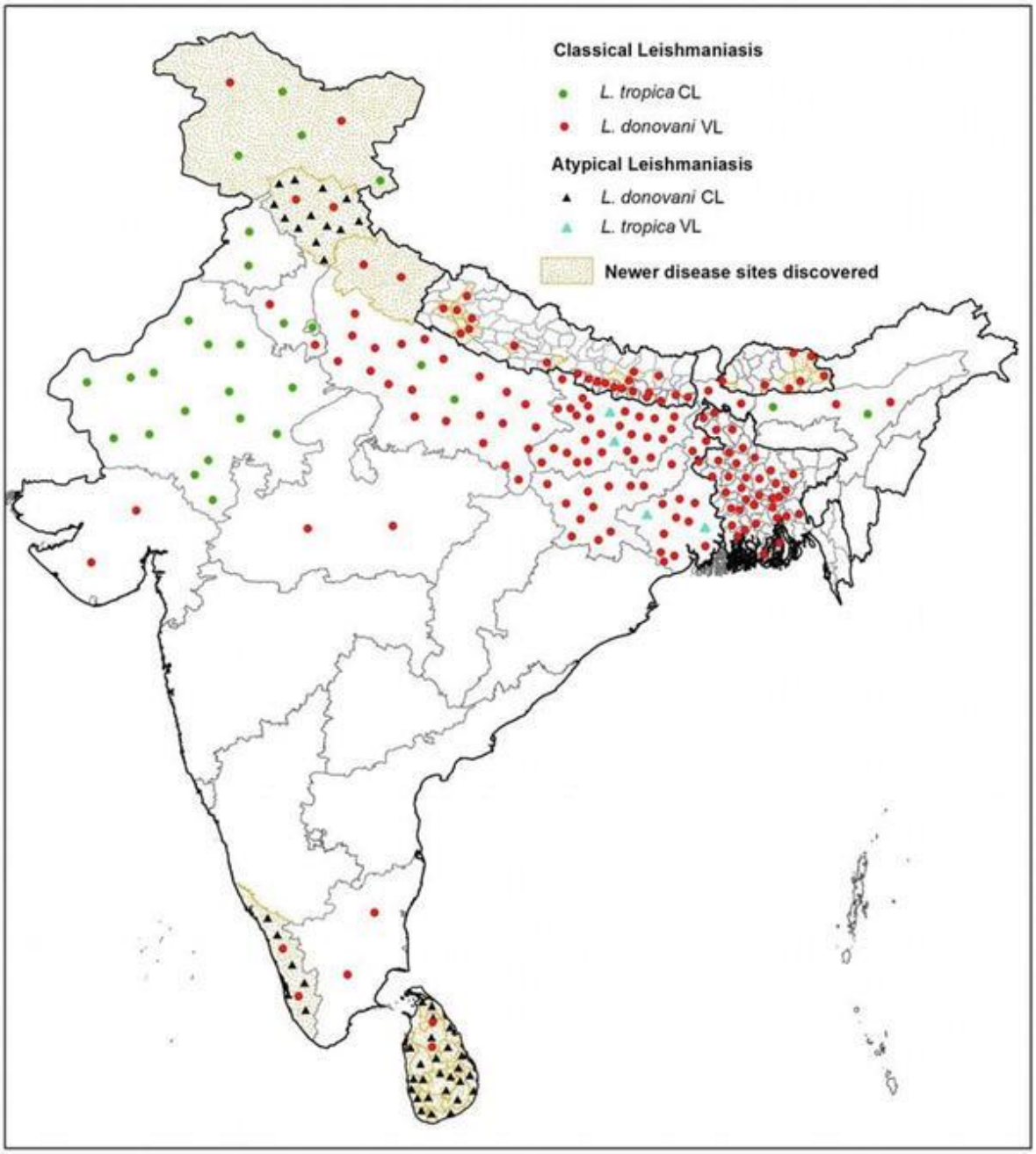

Figure 1.3: Distribution of Leishmaniasis in Indian subcontinent [17].

1.4. Leishmaniasis treatment

Like many other diseases, leishmaniasis possesses treatment options, yet these therapeutics encounter multifaceted challenges, including elevated costs, efficacy concerns, toxicity and potential side effects [20]. Moreover, the rise of drug resistance, coupled with a limited understanding of the interplay between the host and the pathogen, complicates the efficient implementation of treatment strategies. The limited knowledge of immunobiology of the parasite presents a substantial hurdle in the quest for new drugs and vaccines for this disease.

Currently, various treatment options for leishmaniasis remain under scrutiny, awaiting further exploration and insights [21].

1.4.1. Chemotherapy

Chemotherapy is a treatment method for leishmaniasis that utilizes chemical substances, usually in the form of drugs or medications, to treat a disease. These drugs are employed to combat the parasitic infection caused by *Leishmania* species, aiming to eliminate or suppress the growth of the parasites within the host's body [22]. The therapeutic arsenal for leishmaniasis encompasses medications such as pentavalent antimony, amphotericin B (AmpB), miltefosine, paromomycin, and pentamidine [23]. While pentavalent antimony stands as the primary treatment for VL and CL, its notable drawback lies in the occurrence of side effects upon administration. These side effects, including vomiting, nausea, headache, and anorexia, present a challenge in the pursuit of effective and well-tolerated treatments for the disease [24]. In addition to its side effects, resistance to pentavalent antimony has emerged, resulting in therapeutic challenges and treatment failures. Factors contributing to this resistance include a compromised host defense system, the use of unauthorized drug batches, the specific *Leishmania* species involved, and incomplete drug treatment durations [25]. AmpB has risen as a secondary line of treatment. However, the widespread clinical application of AmpB has been restricted owing to its considerable toxicity. This high toxicity level can induce clinical complications such as nephrotoxicity, liver damage, nausea, fever, etc., in patients [26]. The drug's mechanism of action occurs within the cytoplasmic membrane of parasites, where it specifically binds to ergosterol. This interaction facilitates an increase in membrane permeability and promotes ion influx [27]. AmpB stands as the most efficacious treatment presently available for VL in India. Additionally, it serves as a treatment option for individuals with co-infections of VL along with HIV or tuberculosis (TB) [28]. Miltefosine stands as the solitary oral medication effective in treating both VL and CL, with a prescribed treatment duration of 28 days. However, a significant drawback is its prolonged elimination process, resulting in an extended residence time in the body. This extended duration elevates the risk of side effects for patients undergoing the drug regimen [29]. Combined therapeutic approaches involving liposomal AmpB with miltefosine, as well as miltefosine with paromomycin, have been employed for treating VL. This combination strategy has demonstrated enhanced cost-effectiveness compared to the use of miltefosine alone [30]. Paromomycin, an aminoglycoside antibiotic, demonstrates heightened efficacy against CL and is also

employed in the treatment of VL. Despite the current lack of clarity on the precise mechanism underlying paromomycin's action in the *Leishmania* pathogen, its notable capacity for inhibiting various *Leishmania* species is evident [31]. Pentamidine serves as a treatment for VL when resistance develops to primary or secondary therapeutic options. Administered via the intramuscular route, the exact mechanism of action of pentamidine is unclear, but it is known to affect the parasitic mitochondria. However, instances of resistance to pentamidine have been reported in certain cases of leishmaniasis [32]. Table 1.1 provides an overview of various chemotherapeutic drugs, detailing their routes of administration along with their respective advantages and disadvantages.

Table 1.1: Chemotherapeutic drugs for leishmaniasis treatment [20].

Sl. No.	Drug	Route of administration	Pros	Cons
1	Pentavalent antimonial	Intralesional, intramuscular or intravenous	Cheap and easily available	Develop resistance
2	AmpB	Intravenous	Less resistance	Nephrotoxicity
3	Liposomal AmpB	Intravenous	Less toxic	Costly drugs
4	Miltefosine	Oral	Better efficacy	Drug resistance
5	Paromomycin	Intramuscular (VL) or topical (CL)	Low cost	Efficacy varies with species and region
6	Pentamidine	Intramuscular	Small course of treatment	Efficacy of drug changes with species

1.4.2. Immunotherapy

Immunotherapy for leishmaniasis entails leveraging the body's immune system to combat the parasitic infection induced by *Leishmania* species. The objective of this therapeutic approach is to augment or adjust the immune response, facilitating improved control and elimination of the parasites [33]. The primary idea of this therapy is to diminish the adverse effects of the drug on the host and to mitigate the development of resistance. Diverse immunotherapeutic strategies were involved which includes the utilization of cytokines,

immunomodulators, the amalgamation of chemotherapy and immunotherapy, as well as vaccines, have been implemented in the management of both CL and VL [34]. Immunotherapy centered around cytokines has attracted considerable attention due to its potential role in preventing resistance. Key cytokines in this context include Interleukin (IL)-12 and interferon (IFN)-γ, which play pivotal roles in a variety of immunotherapeutic strategies. The intricate balance between pro- and anti-inflammatory cytokines holds paramount importance in the immunopathogenesis of *Leishmania* infection [35]. Immunomodulators in the context of leishmaniasis denote compounds or agents capable of modulating and regulating the immune response in the presence of *Leishmania* parasites. The impact of these immunomodulators on both VL and CL has been investigated, either as standalone interventions or in conjunction with chemotherapeutic agents. This exploration aims to discern the nuanced effects of immunomodulation on the immune system's response to *Leishmania* infection, contributing to a more comprehensive understanding of potential therapeutic strategies [36]. The combined application of chemotherapy and immunotherapy for CL and VL demonstrated superior efficacy compared to individual therapeutic modalities. In murine models, the implementation of a combination therapy approach resulted in a notable reduction in CL lesions. Similarly, for VL, the combination therapy exhibited an augmented rate of recovery [33]. Numerous studies have delved into varied components integrated into vaccine formulations, encompassing specific elements derived from *Leishmania*, to serve as immunotherapeutic agents. Moreover, distinct constituents, such as specific leishmanial components, live parasites, and inactivated parasites, has been explored in diverse research endeavors as immunotherapeutic tools. Several potential vaccine candidates underwent testing in experimental models, including dogs, BALB/c mice, and hamsters, to evaluate their efficacy against *Leishmania* infection [35]. The elicitation of immunity following infection implies that vaccination holds a viable strategy for preventing cutaneous leishmaniasis, both in animal models and human subjects [37].

1.4.3. Nanoparticles therapy

In the medical domain, nanoparticles have emerged as pivotal therapeutic entities. Their application in pathogen elimination involves leveraging inhibitory effects. Nanoparticles, whether utilized independently or in conjunction with other agents, demonstrate the potential to eliminate parasites. Studies have indicated that the co-administration of nanoparticles with drugs enhances efficacy while concurrently mitigating adverse effects [38]. Nanoparticles generally provide various benefits, including reduced toxicity, enhanced

delivery systems, improved solubility for hydrophobic activation, and increased bioavailability. In the treatment of leishmaniasis, different structural nanocomponents were utilized which encompass liposome nanoparticles, polymeric nanoparticles, lipid nanoparticles, as well as TiO_2, Ag_2O, and ZnO nanoparticles [39]. Exploration of nanoparticle-based drug studies on *Leishmania* species has expanded and improved the treatment options for leishmaniasis, paving the way for more effective anti-leishmania drugs.

i. Liposome nanoparticles

Liposomal nanoparticle systems exhibit regulated drug release, imparting heightened treatment efficacy. The adsorption or encapsulation of drugs within nanoparticles showed antileishmanial activity and results in a significant reduction in toxicity, minimizing adverse effects on the host. Additionally, this mechanism facilitates enhanced drug delivery in comparison to conventional formulations in leishmaniasis treatment [40]. Medications employing liposome nanostructures for the treatment of different manifestations of leishmaniasis include liposomes containing hexadecylphosphocholine, liposomal AmpB, bovine serum albumin nanoparticles incorporating AmpB, mannosylated chitosan nanoparticles loaded with curcumin, chitosan nanoparticles conjugated with mannose and lipid formulations of AmpB [39].

ii. Polymeric nanoparticles

Biodegradable polymer nanoparticles find extensive utilization in drug delivery and are also employed for encapsulating essential molecules such as DNA, RNA, and proteins, playing a pivotal role in biomedical applications [41]. Various potential drugs related to leishmaniasis use polymer nanoparticles for showing anti-leishmanial activities. Some of the polymeric based nanoparticles are Primaquine-loaded polyisohexylcyanoacrylate nanoparticles [42], AmpB-loaded nanoparticles [43], mannose-anchored thiolated AmpB nanocarriers [44], mannosylated thiolated paromomycin-loaded PLGA nanoparticles [45], etc.

iii. Lipid nanoparticles

Solid lipid nanoparticles (SLNs) are composed of biocompatible lipids that maintain a solid state at room temperature. These carriers offer benefits such as controlled drug release, improved stability, and the capacity to encapsulate pharmaceutical agents. Conversely,

nanostructured lipid carriers (NLCs) involve a combination of solid and liquid lipids, forming a more flexible matrix with additional features like enhanced drug loading, controlled release, and increased stability compared to SLNs [46]. Both SLNs and NLCs are recognized as second-generation colloid carriers, primarily employed to augment drug delivery. Notably, these nanoparticle carriers have been employed in anti-parasitic drugs to enhance efficacy [47]. The application of conjugated AmpB with SLNs and paromomycin with SLN has been investigated in leishmanial infections, revealing heightened effectiveness [48, 49]. Lipid nanoparticles containing oryzalin, artemisinin-loaded poly lactic co-glycolic acid nanoparticles, biogenic silver nanoparticles, and andrographolide nanoparticles are among the lipid-based nanoparticles utilized in various model studies infected with *Leishmania* species, demonstrating superior anti-leishmanial effectiveness [39].

iv. TiO_2 and Ag_2O nanoparticles

Distinctive physical and chemical properties of titanium dioxide (TiO_2) and silver oxide (Ag_2O) nanoparticles have captivated numerous researchers, prompting their utilization in medicine and therapeutic applications. These two nanoparticles of TiO_2 and Ag_2O was reported to show antimicrobial solution on bacteria and *Leishmania* parasite, thus showed antileishmanial effect [50]. A study that combined application of meglumine antimoniate with TiO_2Ag nanoparticles intensifies its anti-leishmanial efficacy, concurrently mitigating the drug's toxicity [51]. In a research investigation, the proliferation and metabolic activity of *Leishmania* parasites are suppressed by silver nanoparticles. When exposed to UV light, Ag_2O nanoparticles exhibit superior augmented antimicrobial efficacy [52].

1.5. Development of vaccines against Leishmaniasis

Currently, there are no licensed vaccines available for the prevention of leishmaniasis, despite the significant impact of the *Leishmania* parasite on people, including deaths and adverse effects. However, vaccine development efforts have been underway for several decades, aiming to provide long-term protection against this debilitating disease. Leishmaniasis treatment depends on existing anti-leishmanial medications, yet they fall short due to their expense, toxicity, and adverse effects, as well as prolonged dosing regimens with varying levels of effectiveness. Moreover, anti-leishmanial drugs currently face resistance in multiple endemic regions worldwide, exacerbating the situation. Consequently, there is an instant need to create a potential vaccine against leishmaniasis. In this context, various vaccine candidates and strategies have been explored, ranging from live

attenuated and subunit vaccines to DNA and recombinant protein-based approaches. Understanding the challenges and advancements in vaccine development for leishmaniasis is crucial for addressing the unmet medical need posed by this neglected tropical disease. Table 1.2 presents various generations of vaccines, types of vaccines, methods, and expressions used in combating leishmaniasis.

1.5.1. First Generation Vaccines of Leishmaniasis

First-generation vaccines for combating leishmaniasis typically leverage antigens sourced from the entirety of the parasite, whether in an inactivated state or attenuated for live use. The ease and cost-efficiency of cultivating *Leishmania* in cell-free media facilitated the initial adoption of whole inactivated parasites [53]. These pioneering vaccines, originating from early investigations dating back to the late 1930s and 1940s, aimed to instigate protective immune responses against *Leishmania* infections. Notably, a study conducted by Mayrink et al. in Brazil delved into the development and evaluation of a first-generation vaccine against leishmaniasis, yielding satisfactory outcomes albeit with only a 50% protective effect [54]. Further research in Brazil underscored the efficacy of a combined treatment approach involving killed *L. amazonensis* paired with a partial dose of meglumine antimoniate for addressing CL [55]. Additionally, investigations in Ecuador revealed promising protection against CL through the utilization of a formulation comprising *L. brasiliensis, L. guianensis,* and *L. amazonensis* [56]. In Venezuela, a combination of adjuvant Bacillus Calmette-Guérin (BCG) with killed *L. amazonensis* was employed to treat CL [57]. Furthermore, the adjunctive application of BCG alongside killed *Leishmania* vaccines was investigated to bolster the cell-mediated response in experimental models [58]. In Iran and Sudan, examinations have assessed a killed vaccine derived from *L. major,* manufactured by the Razi Institute [58,59]. These inquiries highlight the potential of employing a killed *Leishmania* vaccine, with or without BCG as an adjuvant, as a safe alternative [60]. The safety aspect of killed vaccines underscores their favorable profile in terms of safety, rendering them a promising avenue for further exploration in vaccine development endeavors.

Live-attenuated vaccines involve intentionally removing genes essential for an organism's virulence and survival. This modification enables them to stimulate an immune response similar to a natural infection. Although live-attenuated vaccines closely mimic natural infection and effectively induce protective immunity, concerns about safety hinder their full

potential benefits. Existence of antibiotic-resistant genes, and the risks involved in vaccinating immunosuppressed individuals are significant factors to address. Vaccines derived from *L. major, L. mexicana, L. braziliensis,* and *L. donovani* have been created, demonstrating notable efficacy in safeguarding susceptible mice against CL and VL [61, 62,63]. A genetically modified strain of *L. donovani* was developed through the deletion of the gene responsible for Ldp27. When immunized with these Ldp27-deficient parasites, subjects exhibited diminished parasitic burdens and experienced improved long-term Th1-mediated protection against challenges of CL and VL [64].

1.5.2. Second generation vaccines of Leishmaniasis

Second-generation vaccines center on pathogen antigens, comprising purified fractions of native proteins, genetically engineered subunits. These vaccines consist of precisely defined molecular constituents, employing recombinant antigens—potentially one or more proteins—and designated adjuvants [65]. In the context of *Leishmania*, factors such as antigenic variation, antigen conservation among different species, and accessibility of the *Leishmania* genome sequence are taken into account. This has allowed for the utilization of *Leishmania* proteins as potential vaccine applicants [66]. The approach relies on utilizing recombinant subunits alongside modified *Leishmania* strains, recombinant bacteria or viruses harboring genes encoding *Leishmania* antigens [67]. The Infectious Disease Research Institute (IDRI) reached a significant milestone by developing LEISH-F1, a pioneering recombinant vaccine candidate that successfully advanced to clinical trials. Additionally, IDRI has developed candidates like LIESH-F2 and LEISH-F3, both of which have progressed to clinical trials, showcasing encouraging outcomes [68]. Studies have investigated the potential of utilizing sand fly salivary proteins as a standalone vaccine against leishmaniasis, or in combination with *Leishmania* proteins, to create a potent vaccine candidate. Research suggests that individual saliva antigens may exert paradoxical impacts on disease outcomes when compared to whole saliva. Additionally, findings have revealed that salivary proteins can independently initiate the cell-mediated immune response, unlike recombinant protein candidates that often rely on adjuvants for activation [69].

1.5.3. Third generation vaccines of Leishmaniasis

As third-generation vaccines, genetic immunization strategies have emerged, utilizing the direct administration of nucleic acids, including mRNA, naked plasmid DNA, or

encapsulated within a viral vector. Third-generation vaccines surpass second-generation counterparts by providing improved protein stability, leading to a significantly elevated level of protection. In murine models, an RNA vaccine expressing LEISH-F2 followed by a subunit vaccine demonstrated effectiveness against *L. donovani* [70]. These vaccines, containing plasmid DNA, prompt the synthesis of endogenous proteins post-injection, thus eliciting targeted immune responses. They facilitate the development of both cellular and humoral immunity and are offered in various formats, such as recombinant proteins, single vaccines, or multigene formulations [71, 72]. In the realm of *Leishmania* research, a broad spectrum of antigens has been explored, and commonly used DNA vaccines primarily consist of protein components. Currently, pre-clinical trials for the development of leishmaniasis vaccines involved testing on mouse models infected with *L. major, L. mexicana,* and *L. amazonensis*. Moreover, in the evaluation of vaccines, strains of VL including *L. donovani, L. infantum*, and *L. chagasi* were utilized in various animal models. [65]. The initial DNA vaccine designed for leishmaniasis featured the gene encoding gp63, which has demonstrated its ability to induce a Th1 response and offer substantial protection against CL due to *L. major*. LACK, another DNA vaccine extensively studied, offers diverse levels of protection against infections caused by *L. major, L. donovani,* and *L. infantum* in mouse and dog models [73,74]. DNA vaccines are attractive because of their simple manufacturing process, stability, and various safety characteristics. Moreover, they are acknowledged for their ability to trigger stronger Th1 immune responses in contrast to protein-based vaccines. Despite demonstrating efficacy in animal models, DNA vaccines have faced challenges in clinical trials, particularly in terms of their immunogenicity and clinical benefits. This has made their translation to human use challenging [75, 76]. Currently, there are no approved DNA vaccines for human use, including those targeting leishmaniasis and other infections.

Table 1.2: Vaccination strategies investigated for combatting *Leishmania* [77].

Sl. No.	Generation	Type of vaccine	Methods	Expression
1	First generation vaccine	Killed *Leishmania*	Formulations of polyvalent	CL
			BCG as adjuvant	CL
		Live attenuated	Biopterin transporter 1 (BT1)-deleted *L. donovani*	VL
			Ldp27 gene deleted *L. donovani*	VL
			(HSP)-70-II null *L. infantum*	VL and CL
			L. infantum KHARON1 (KH1) null	VL
2	Second generation vaccine	Subunit and recombinant vaccines	LEISH-F1, LIESH- F2 and LEISH-F3	VL and CL
			LJM19 and LJL143	VL and CL
			PdSP15	VL and CL
3	Third generation vaccine	DNA vaccines	LEISH-F2	VL
			gp63	VL and CL
			LACK	VL and CL

1.6. Drug resistance in Leishmaniasis

Leishmaniasis, a neglected tropical disease endemic in over 98 countries. Treatment failure in patients with Leishmaniasis is a frequent occurrence, as evidenced by cases where the same species of *Leishmania* causing identical clinical manifestations exhibit varying responses to the same drugs, ultimately resulting in treatment inefficacy for affected individuals [78]. Treatment failure in Leishmaniasis can stem from a myriad of reasons, ranging from issues with the drugs themselves to complications like co-infections such as HIV or the presence of *Leishmania* RNA virus. Parasite-related factors encompass various elements, such as the inherent virulence of the specific strain of *Leishmania* causing the infection. Furthermore, leishmaniasis caused by RNA viruses can incite distinct host immune responses. Moreover, treatment failure can occur when individuals with

leishmaniasis exhibit favorable responses to a specific therapy for similar clinical manifestations, but this efficacy is not observed universally among all patients. This scenario arises when patients are infected with different *Leishmania* species or strains [79]. Additionally, factors related to the host, like compromised immunity or incorrect dosages, can also contribute [80]. Moreover, the emergence of drug resistance poses a significant challenge in effectively treating this disease.

Drug resistance in Leishmaniasis emerges as the *Leishmania* parasites evolve mechanisms to resist the impact of anti-leishmanial medications, undermining the effectiveness of treatment plans. Across different regions and in the treatment of VL, a variety of antileishmanial drugs are employed. Notably, pentavalent antimonial compounds have historically been administered to treat VL in areas such as Africa, South America, India and Nepal. Conversely, in the Mediterranean basin, liposomal AmpB has been the preferred choice [81]. From many decades, pentavalent antimonials, including sodium stibogluconate and meglumine antimonate, continue to be the primary choice for treating VL. This preference stems from studies demonstrating the heightened sensitivity of *L. donovani* and *L. brasiliensis* to sodium stibogluconate compared to other *Leishmania* species, coupled with their extensive use over two decades [82]. However, the emergence of resistance to pentavalent antimonials was first identified in North Bihar in 1980s, where approximately a quarter of individuals exhibited unresponsiveness to these medications [83].

Both antimony and arsenic are heavy metals, sharing certain characteristics. In the northeastern regions of India, it has been noted that *Leishmania* parasites exhibit reduced responsiveness to antimonial drugs. This phenomenon is attributed to the high levels of arsenic present in the groundwater of this area. Consequently, arsenic exposure to the *Leishmania* pathogen has led to the growth of antimony resistance in certain regions of India. Additionally, individuals afflicted with leishmaniasis residing in areas with lower arsenic levels demonstrate a more favorable response to antimonial drugs [84]. Research has revealed that resistant parasites exhibit lower levels of antimony compared to their sensitive counterparts. Increased expression of the Aquaporin 1 membrane carrier (AQP1) enhances parasite sensitivity to antimonial drugs, whereas decreased levels of AQP1 lead to reduced responsiveness and eventual resistance. Furthermore, deletion or mutation of the AQP1 gene confers resistance to antimonial upon parasite exposure [85,86]. For pentavalent antimony to exert its action within the parasite, it must undergo reduction to its trivalent form. Diminished biological reduction of antimony results in reduced drug uptake, thereby

promoting resistance. Additionally, overexpression of ATP-binding cassette (ABC) transporters such as ABCI4 and ABCG2 facilitates drug efflux, contributing to antimonial resistance [87,88].

Miltefosine (MIL), introduced in India in 2002, emerged as a promising alternative to pentavalent antimonials due to its high cure rates against leishmaniasis [89]. Miltefosine interferes with phospholipid synthesis and metabolism. Furthermore, it facilitates parasite cell death while having a minimal impact on the host, thus enhancing its therapeutic efficacy [90]. Following a decade since its introduction, the efficacy of MIL has shown a notable decline, transitioning from approximately 90% efficacy during its initial implementation to a range of 10-20% in parasites, consequently resulting in treatment failures. Observations indicate that MIL necessitates approximately 120 hours to eliminate half of the administered doses, which is a considerable high duration [91]. Consequently, its prolonged presence in the body exposes individuals to potential side effects and increases the likelihood of resistance development. The *Leishmania* miltefosine transporter (LMT and/or LRos3) serves as the primary mechanism for the uptake of MIL within the parasite. Experimental evidence from both *in vitro* and *in vivo* studies has demonstrated that mutations or deletions occurring in the LMT gene result in an increase in parasite resistance [92]. This resistance mechanism is attributed to either a diminished uptake or an augmented efflux of MIL. Moreover, elevated expression of ABC transporters also contributes to resistance [93].

AmpB derived from *Streptomyces nodosus*, has been a stalwart in antileishmanial treatment, with a rich history spanning six to seven decades of use against fungal infections [94]. One of its distinguishing characteristics lies in its unique ability to selectively bind to ergosterol present in the cell membranes of *Leishmania* pathogen. This selective targeting sets it apart, as host cells predominantly contain cholesterol in their cell membranes, rendering the drug less sensitive to the host organism [95]. In the realm of leishmaniasis therapeutics, the specter of resistance looms large, even for stalwarts like AmpB. Instances of resistance have surfaced, particularly when the multidrug resistance MDR1 gene is found to be upregulated in resistant strains, indicating a heightened efflux of the drug [96]. Additionally, the involvement of ATP-binding cassette transporters, as well as amplification of the thiol pathway and alterations in membrane composition, have been implicated in promoting resistance within *Leishmania donovani* [96].

The evident vulnerability of monotherapy or single drug treatment to prompt resistance in leishmaniasis prompted researchers to introduce combination therapy [97]. This innovative approach involves administering limited doses of two drugs simultaneously. However, it was demonstrated that even combinations like MIL and paromomycin, as well as SSG and paromomycin, under experimental conditions, showed signs of resistance in both promastigotes and amastigotes [98].

1.7. Drug discovery approaches against Leishmaniasis

Various diseases pose significant challenges to public health, contributing to high mortality rates. In the realm of drug discovery, identifying effective drug targets for these diverse diseases becomes paramount in addressing the associated mortality. In the context of mortality associated with various diseases, drug discovery efforts aim to develop therapeutic interventions that can mitigate the impact of these conditions on human health [99]. Structural biology, system biology and bioinformatics can identify and validate potential drug targets which latter on leads to drug discovery [100]. Bioinformatics plays a crucial role in drug discovery by utilizing computational methods to analyze biological data, including genomics, proteomics, and other omics data [101]. Structural biology contributes to drug discovery by providing insights into the three-dimensional structures of biological molecules, such as proteins and enzymes [102]. Once identified, these drug targets become the focal point for designing and testing new drug molecules. The integration of bioinformatics, structural biology, and advanced computational approaches accelerates the drug discovery process, offering hope for more effective treatments and better outcomes for individuals affected by diverse diseases [103]. Drug targets are often proteins, that are involved in disease processes and can be modulated by drugs to achieve a therapeutic effect. Potential drug molecules are chemical compounds that have the potential to interact with these targets and exert a therapeutic effect.

1.8. Multitarget approach

The multitarget approach in drug development involves designing compounds or therapies that act on multiple targets or pathways within a biological system to achieve enhanced efficacy and therapeutic outcomes. This strategy acknowledges the complexity of diseases and aims to address various disease mechanisms simultaneously, thereby reducing the likelihood of resistance development and improving treatment effectiveness [104]. Observations have revealed that single-targeted drugs tend to develop resistance more

rapidly compared to multitarget drugs. Over recent decades, an increasing number of pathogens have demonstrated resistance to specific antibiotics [105]. Antibiotics with dual or multiple targets exert their action through two mechanisms: firstly, by targeting closely related proteins within the same pathway, and secondly, by binding to distinct molecules involved in separate biological functions. This multifaceted approach enhances effectiveness and mitigates resistance development in pathogens [106]. Nevertheless, there are also numerous antibiotics that interact with two or more discrete molecules that are deemed essential for the treatment of certain diseases. Among the multitarget drugs utilized against diverse pathogens are penicillin, targeting penicillin-binding proteins [107]; ciprofloxacin, impacting both topoisomerase II and IV [108]; tetracycline, which acts on ribosomes and membranes [109]; polymyxin B, targeting both outer and inner membranes [110]; and tyrocidine, believed to affect the membrane and potentially DNA [111]. Miltefosine, a cornerstone in leishmaniasis treatment, is a prime example of a multitarget drug. It influences calcium homeostasis, the replication machinery, and mitochondrial functions, including cytochrome c oxidase activity and mitochondrial membrane integrity, while also impacting metacaspase production [112]. Cavalli and Bolognesi endeavored to identify fresh lead candidates for *Leishmania* and *Trypanosoma* utilizing multitarget ligands [113]. Overall, the multitarget approach shows considerable potential in treating a variety of diseases, including leishmaniasis, by potentially enhancing efficacy, minimizing the risk of resistance emergence, and ultimately improving patient outcomes.

1.9. Drug repurposing approach

Drug repurposing, represents an avenue of drug discovery wherein existing drugs, utilized for different therapeutic purposes, are reconsidered for novel indications, it is also considered as drug repositioning. It constitutes a retrospective approach, leveraging the established safety profiles and known mechanisms of action of these drugs to explore potential alternative targets for specific diseases [114]. Rather than creating entirely new medications from the ground up, drug repurposing capitalizes on the established safety profiles, pharmacokinetics, and other characteristics of existing drugs [115]. Repurposed drugs, having undergone preclinical and early-stage trials, decrease the risk of failure, particularly concerning safety in subsequent efficacy trials. This development process aids in reducing the time required for preclinical testing and ensures thorough safety assessments. [116]. Although investment requirements differ, there are potential cost savings in preclinical and early phase expenses. Although regulatory and later phase costs are more as

it was essential and crucial part similar to noble drug development, drug repurposing ultimately offers a more streamlined and cost-efficient method for introducing treatments for new indications [117].

Various computational and experimental methodologies, either independently or in synergy, contribute to the drug repurposing process. Computational strategies include signature matching, which compares specific drug characteristics with those of other drugs [118]; molecular docking, predicting binding interactions between a molecule and its target protein [119]; genome-wide association studies, identifying genes associated with a disease [120]; and pathway/network mapping, analyzing genetic and protein targets related to a disease [121]. Moreover, retrospective clinical analysis involves systematic scrutiny of electronic health records and clinical trial data [122], while novel data sources encompass large-scale *in vitro* drug screens coupled with genomic data and electronic health record databases [123]. Experimental approaches encompass phenotypic screening, employing *in vitro* or *in vivo* disease models [124], and binding assays utilizing techniques such as affinity chromatography and mass spectrometry to identify relevant target interactions.

Several successful examples of drug repurposing have been observed across various disease treatments. Zidovudine, initially employed in cancer therapy, was repurposed for HIV/AIDS treatment in 1987. Atomoxetine, originally indicated for Parkinson's disease, found utility in managing attention deficit hyperactivity disorder in 2002. Rituximab, utilized in cancer treatment, was later repurposed for Rheumatoid arthritis in 2006. Topiramate, initially prescribed for epilepsy, demonstrated efficacy in treating obesity in 2012. Ketoconazole, employed for fungal infections, was repurposed for Cushing syndrome in 2014. Sildenafil, initially intended for angina, was repurposed for erectile dysfunction in 1998 [117].

In the realm of drug discovery, Law et al. demonstrated the success of employing drug repurposing techniques, showcasing their effectiveness in picking out potential drug contender for treating a spectrum of diseases, ranging from cancers to influenza [125]. Recognizing the pressing challenge of drug resistance, Liu et al. emphasized a strategic solution—repurposing non-antibiotic drugs for diseases experiencing resistance. This approach not only holds promise for overcoming resistance but also lays the groundwork for the development of advanced combinational therapies, aiming to enhance efficacy and broaden treatment options [126]. Josef Jampilek pointed out that drug development is a complex process fraught with numerous obstacles, leading to a substantial failure rate and

prolonged timelines. In addressing this formidable challenge, repurposing existing drugs for antimicrobial purposes emerges as a promising strategy for effectively treating infections. This approach leverages established drugs to expedite the development of treatments for antimicrobial indications, potentially providing a more effictive and practical different to traditional drug development methods [127]. Cheng et al. underscored the escalating challenge posed by multidrug-resistant (MDR) pathogens and emerging viruses. To effectively address this issue, they emphasized the importance of drug repurposing and drug combination screens. These strategies not only improve efficacy but also decrease the toxicity of combined drugs within the host [128]. Overall, drug repurposing represents a valuable strategy for maximizing the therapeutic potential of existing drugs, ultimately benefiting patients by providing innovative treatment options and potentially improving healthcare outcomes.

1.10. Subtractive genomics approach

Identifying drug targets represents a critical juncture in drug discovery. Leveraging the abundant genomic and proteomic data available across diverse sequence databases for both pathogens and hosts has streamlined the process of identifying drug targets for pathogens. Within this context, the subtractive genomic approach has emerged as a valuable tool. This bioinformatics methodology entails comparing genomes of distinct organisms, typically a pathogenic organism and its host or closely related counterparts, to discern genetic features unique to the pathogen [129]. At present, comparative and subtractive genomics are widely used to discover new targets. It aimed at crafting antimicrobial agents and vaccines against pathogens that are resistant to current therapies or lacking appropriate vaccine options. This approach aids in ascertaining the indispensability of these genes or proteins for pathogen survival and their absence in non-host homologues [130]. The overarching objective is to pinpoint potential drug targets or vaccine candidates specific to the pathogen, facilitating the development of targeted therapeutic strategies.

Recognizing the significance of this approach, George and Umrania reported its instrumental role in identifying possible therapeutic targets and uncovering drug-like compounds for *Streptococcus agalactiae*, *S. pneumoniae*, and *S. pyogenes*. This becomes particularly crucial in the face of emerging resistance observed in these bacterial strains [131]. Subtractive genomics has proven to be a dominant method for identifying new therapeutic drug targets, as evidenced by the comprehensive analysis of the entire proteome of

Mycoplasma genitalium. This process opens up avenues for designing potent drugs against the pathogen, highlighting the potential for innovative therapeutic interventions [132]. Recognizing the need to address Campylobacter infections in young children, Mehla and Ramana strategically employed subtractive genomics and metabolic pathway strategies. These approaches played a pivotal role in identifying novel drug targets against *Campylobacter jejuni*, the causative pathogen behind the infection, presenting a promising avenue for combating this health concern [133]. The subtractive genomic approach has been effectively utilized to identify new therapeutic targets and vaccine candidates for various pathogens, including bacteria, viruses, and parasites. It offers a systematic and efficient way to uncover unique features of pathogens that can be exploited for therapeutic intervention.

1.11. Scope of the work

Despite decades of research, effective treatments for leishmaniasis are still limited, and the exposure of drug resistance has become a major challenge. The development of new drugs against *L. donovani* is imperative to combat the disease effectively and address the growing threat of drug resistance. With the ongoing emergence of resistance, the pathogenicity of Leishmaniasis is increasing, underscoring the need for new targets and potent ligands to counteract this trend. The scope of work on drug discovery for *L. donovani* and other *Leishmania* species underscores a multidisciplinary approach that integrates diverse scientific disciplines, encompassing multitarget strategies, drug repurposing, and subtractive genomics. The research endeavors to bridge gaps in our current understanding of drug development for leishmaniasis is to introduce innovative computational techniques. Our focus areas include identifying novel drug targets, screening compound libraries at high throughput, optimizing lead compounds, and assessing the binding affinity and stability of protein-ligand complexes. Our work focuses on five specific drug targets: adenine phosphoribosyl-transferase (APRT), a component of the purine pathway; dihydroorotate dehydrogenase (DHODH), involved in the pyrimidine pathway; pyridoxal kinase (PK), part of the vitamin B6 pathway; sterol alpha-14 demethylase (SDM), integral to sterol biosynthesis; and glutamate dehydrogenase (GDH). Throughout our study, we emphasize the meticulous selection of these protein targets and the application of computational techniques with diverse methodologies.

The central aim of the thesis is to advance and implement innovative methods that offer a more realistic and nuanced approach to target fishing and drug identification, particularly in

the context of a distinct class of diseases. By concentrating on leishmaniasis, the thesis contributes to the broader landscape of infectious diseases, offering insights and methodologies that may have implications for similar protozoan-driven ailments. The interdisciplinary nature of the research, combining various methods, underscores the commitment to advancing the field and potentially paving the way for more efficacious treatments for leishmaniasis. By enhancing our knowledge of parasite's biology and capitalizing on emerging therapeutic targets, we have the potential to create efficient treatments to alleviate the impact of leishmaniasis and enhance public health worldwide.

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