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Review Article

Recent Advancements in Drug Discovery Models for Visceral Leishmaniasis

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ABSTRACT

The aim of present study was to report an herbal antidandruff shampoo utilizing plants that are well-reported for their cleansing effects on hair. These natural plant components are safer as compared to synthetic chemicals (e.g. silicones and polyquaterniums), used as conditioning agents. Present study utilizes flowers of *hibiscus rosa sinensis*, seeds of *trigonella foenum-graecum* (fenugreek), fresh leaves of *murraya koenigii* (curry patta), fruits of *Acacia concinna* (shikakai), fruits of *sapindus mukorossi* (reetha) and dried seed of *Cyamopsis tetragonoloba* (guar gum). Dried powder forms of all these plants were used. An exhaustive evaluation of prepared formulations was also reported. The evaluation studies ensure the antidandruff potential of reported formulations. Furthermore, study showed good foam foaming, rinsing, and good dirt dispersion activity; yet, extra research are proposed to improve the product's overall quality.

INTRODUCTION

The term "tropical diseases" describes illnesses that are more common in the world's tropical regions. These primarily consist of infectious diseases that are common in hot, humid tropical regions, such as African trypanosomiasis, Chagas disease, dengue, filariasis, leishmaniasis, malaria, onchocerciasis, and schistosomiasis. African trypanosomiasis, leishmaniasis, malaria, and Chagas disease are all brought on by infections with protozoan parasite diseases. Over 500 million people worldwide are afflicted by these protozoan parasite illnesses, which cause high rates of mortality and morbidity (WHO, 2016). In tropical and subtropical nations, protozoan parasites such as *Leishmania* spp., *Plasmodium* spp., and *Trypanosoma* spp. are more common and cause significant mortality and diminished working capacity [1]. Few antiparasitic medications have been created in recent years, despite the fact that diseases brought on by parasitic protozoa pose a serious threat to humankind [2]. Many laboratories and pharmaceutical companies have shown unprecedented interest in new antiparasitic drug discovery programs in the development of approaches to treat and control malaria and other neglected diseases [3]. These diseases are among the 17 diseases collectively referred to as neglected tropical diseases (NTDs), which largely affect the poorest citizens of 149 nations and territories. More than 1 billion individuals suffer from one or more NTDs, and over 2 billion people are at risk of getting one, according to recent World Health Organization (WHO)

estimates. Every year, an NTD is thought to kill about 534,000 people. The overall number of fatalities from NTD plus malaria is one million if we include the 438,000 deaths from malaria per year. These diseases still need vaccines. Therefore, medication therapies are the primary means of curing these illnesses.

Leishmaniasis and malaria can only be treated with a small number of medications. Due to the development of resistance, the majority of them are hazardous and are leaving the market. The development of novel medications and drug leads is required due to the toxicity and growing resistance to existing antileishmanial and antimalarial medications. Both *in vivo* and *in vivo* screening methods aids in the selection of novel pharmacophores from libraries of synthetic and natural chemicals forms the basis of drug discovery strategies for both disorders. Analyzing the proliferation of intracellular parasites is extremely difficult because both leishmania and malaria parasites occur in intracellular forms in the human host [4]. It is difficult to maintain intracellular versions of these parasites *in vitro*. The parasite's growth within the host cells can be examined using a variety of methods, and the host and parasite cells must be kept together. The technologies that are now available are labor-intensive and have significant limitations. There is an urgent demand for simpler technologies that produce repeatable outcomes. This article presents the current state of the available screening models for visceral leishmaniasis.

VISCERAL LEISHMANIASIS

Visceral leishmaniasis (VL) is caused by the protozoan parasite Leishmania donovani. If untreated, VL is lethal. In East Africa and the Indian subcontinent, it is extremely endemic. Between 200,000 and 400,000 new cases of VL are thought to arise annually around the world. According to WHO (2010) and the WHO Expert Committee on the Control of the Leishmaniases, more than 90% of new cases occur in six countries: Bangladesh, India, Brazil, Ethiopia, Sudan and South Sudan.

TREATMENT OF VL: LIMITATIONS AND PROBLEMS WITH CURRENT THERAPEUTIC OPTIONS

Even the few medications that are currently available to treat leishmaniasis have limited effectiveness and notably substantial toxicities at therapeutic doses (Table 1).

Table 1: Drugs commonly used in the treatment of VL.

Drug	Mode of Action	Toxicity
Amphotericin B	Ergosterol pathway	Severe Kidney damage
Sodium Stibogluconate	Multiple Pathways	Pancreatitis, Cardiac toxicity
Miltefosine	Lipid metabolism, Apoptotic effect	Teratogenicity
Azole	Ergosterol pathway	Cardiac problems

Due to rising multiple drug resistance, certain first-line therapy medications, such as sodium stibogluconate, have already lost their effectiveness [5]. Meglumine antimonite and sodium stibogluconate (SSG), two pentavalent antimony compounds, continue to be the cornerstones of VL treatment [6]. However, the use of amphotericin B has become the primary therapeutic choice in a number of places where leishmaniasis has become resistant to antimonials [7]. Both amphotericin B and antimonials are extremely toxic, require a lengthy course of treatment, and must be given parenterally [5]. However, lipid formulations of amphotericin B have significantly decreased the drug's toxicity, enabling the administration of high dosages and reducing the length of treatment. Though, the drug's usage in endemic areas and for routine treatments has been restricted because to the expensive expense of treatment and the need for supervised hospitalization [8]. Miltefosine, an alkyl phospholipid derivative that was initially created as an anticancer medication, was recently authorized as India's first oral medication to treat visceral leishmaniasis. Miltefosine is > 90% curative for cutaneous disease in Colombia and visceral disease in India when taken at a dose of about 2.5 mg/kg/day for 28 days [9]. Miltefosine causes kidney damage and reversible gastrointestinal problems. Pregnant women and other

young females who do not take contraceptives cannot use this medication due to its teratogenic nature [9]. The antibacterial/antiprotozoal aminoglycoside paromomycin was found to have antileishmanial properties as early as the 1960s. Due to the end of its manufacture and the lack of appropriate formulations, the medicine continued to be disregarded. Phase III clinical studies showed that injectable paromomycin was just as effective as amphotericin B in treating visceral leishmaniasis [10]. The azole chemicals, which are antifungal, show promise as antileishmanial drugs. However, the outcomes of using antifungal azole medications to treat VL have been somewhat inconsistent. In the past, pentamidine isethionate, an aromatic diamidine, was administered parenterally as a second-line treatment for leishmaniasis; however, subsequent trials did not yield a satisfactory cure. Pentamidine's low effectiveness and additional safety issues have led to its discontinuation as a treatment for visceral leishmaniasis [6].

As a result, the available medications for treating VL are quite limited, which emphasizes the necessity of finding novel antileishmanial medications with established clinical efficacy and wider applicability for oral treatment of VL. Table 1 lists the main clinical medications along with their method of action, primary source, available toxicity, and key notes. Figure 1 provides the structures.

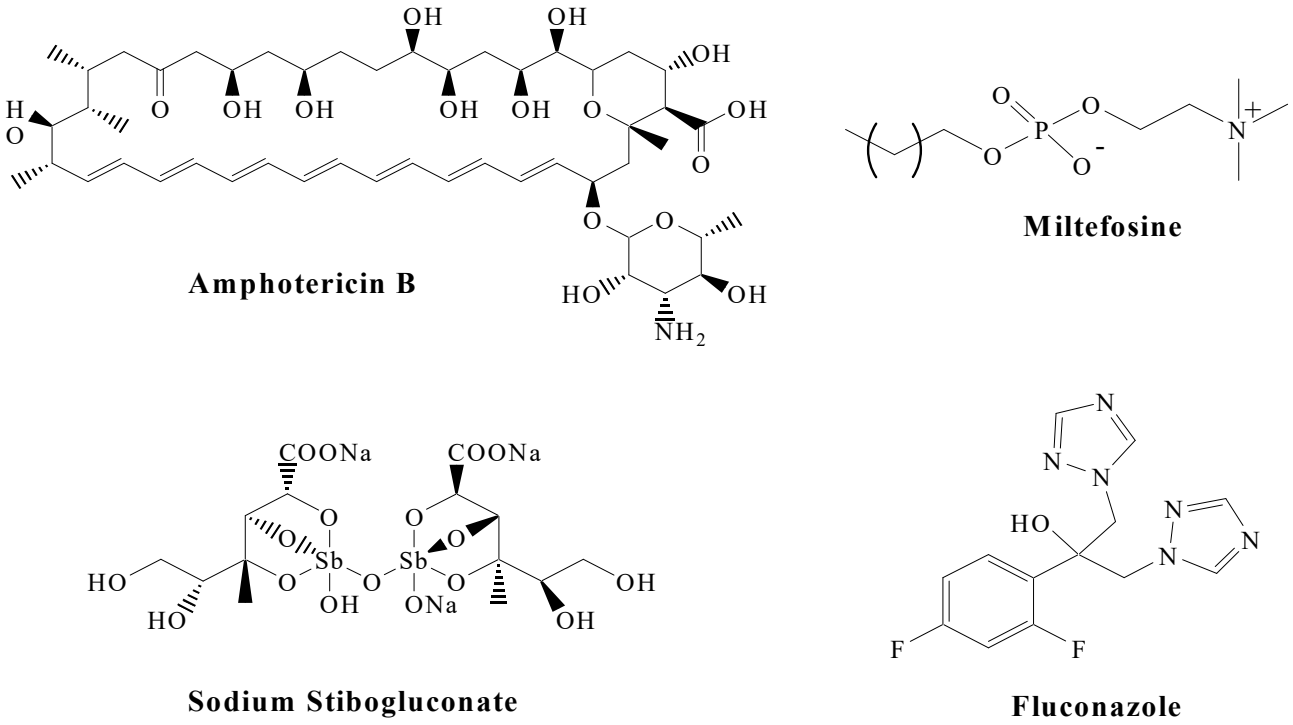

Fig. 1. Chemical Structures of drugs primary used in VL.

ANTILEISHMANIAL DRUGS CURRENTLY UNDER CLINICAL DEVELOPMENT FOR VL.

Nitroimidazoles

Fexinidazole

It is thought that fexinidazole functions as a prodrug that needs to be reduced by nitroreductases in order to produce cytotoxic species that harm proteins, lipids, and DNA [11]. A study was conducted to evaluate the leishmanicidal activity and preclinical profile of fexinidazole since leishmania parasite genomes possess a homologous nitroreductase gene. Five daily doses of 200 mg/kg in BALB/c mice suppressed infection by 98.4%, demonstrating the drug's exceptional *in vivo* sensitivity in *L. donovani*. In the murine model of infection, lower dosages of the medication were equally successful; the estimated ED50 and ED90 were 12 and 57 mg/kg, respectively [12]. The

leishmanial homolog of this nitroreductase was overexpressed in *L. donovani*, increasing sensitivity to fexinidazole sulfone by a factor of 19. This suggests that nitroreductase was essential for activating fexinidazole and its metabolites in *L. donovani* [12]. Fexinidazole has been the subject of a phase 1 trial in healthy male volunteers [13,14]. The effectiveness of fexinidazole at a daily dose of 1800 mg (3 tablets) once daily for four days, followed by 1200 mg (2 tablets) once daily for six days, is being investigated in a phase II proof of concept trial for VL patients in Sudan [15].

Pretomanid (Pa-824)

PA-824 is a bicyclic compound that resembles nitroimidazole. With EC50 values of 0.9± 0.1 and 4.9± 0.3μM against promastigotes and intracellular amastigotes, respectively, (S)-PA-824 demonstrated antileishmanial activity against both phases of the parasite's development [16]. Miltefosine (68.7% suppression) and sodium stibogluconate (41.9%) were found to be less effective than (R)-PA-824 (99.9% suppression) at this dosage [16]. 50, 250, 500, 750, 1,000, 1,250, or 1,500 mg of (S)-PA-824 or multiple doses of 200, 600, and 1,000 mg daily for seven days were administered orally to 58 healthy male volunteers in a phase 1 study [17]. Pharmacokinetic characteristics were in line with a once-daily regimen, and PA-824 was well tolerated after oral dosages once daily for up to seven days.

Quinoline Derivatives

8-Aminoquinolines

The Walter Reed Army Institute of Research (WRAIR, USA) found 8-aminoquinoline analogue sitamaquine (WR-6026), that is being explored as an oral therapy for VL. In hamsters, sitamaquine was found to be 708 times more effective against *L. donovani* than meglumine antimoniate (Glucantime®) [18]. In Kenya, the first phase 2 research involved 16 patients treated with 0.75–1.00 mg/kg for 2-4 weeks, with a 50% cure rate after 28 days of 1 mg/kg treatment [19]. At different dosages of sitamaquine, respectively, 92 of 106 (87%) Brazilian patients experienced a final cure (primary efficacy outcome), while 25 of 31 (81%), 24 of 27 (89%), 23 of 23 (100%), and 20 of 25 (80%) patients experienced a cure [20]. According to recent studies, leishmania parasites accumulate sitamaquine [21]. Its molecular targets are still unclear, though. According to Loiseau et al. sitamaquine's brief elimination half-life is halting the emergence of resistance [22]. A decision for further development is based on the laboratory selection of a sitamaquine-resistant clone of *L. donovani* and the phase II clinical trials that highlight certain negative effects as methemoglobinemia and nephrotoxicity [20].

IN VIVO PHENOTYPIC MODELS FOR ANTILEISHMANIAL SCREENING

New antileishmanial drug discovery is characterized by *in vivo* preclinical evaluation and phenotypic parasite culture-based *in vitro* screening [23]. Various techniques for leishmania phenotypic cell-based screening tests have been documented. The types of parasite forms used, the intricacy of the experimental procedure, and the techniques for assessing the development and multiplication of the parasite cells in culture are all different in these experiments. *L. donovani* alternates between the sand fly's digestive system and the phagolysosomes of mammalian macrophages [24]. The parasite takes on two different forms throughout its life cycle. "Amastigotes" are present in the mammalian host, while "Promastigotes" are located in the sandfly host, which serves as a vector for leishmaniasis transmission. Amastigotes are ovoid, intracellular, non-motile stages, while promastigotes are extracellular, elongated, and motile. The infected female sandfly bites the mammalian host, injecting promastigotes into the skin. Promastigotes become intracellular amastigotes after specifically invading macrophage cells. Throughout the mammalian phase of its life cycle, the parasite stays in amastigote form. Sandflies consume amastigotes while blood-feeding on an infected mammal host. During the life cycle's vector phase, the amastigotes change into promastigotes (Figure 2). There have been descriptions of phenotypic screening techniques that use both promastigotes and amastigotes of the parasite.

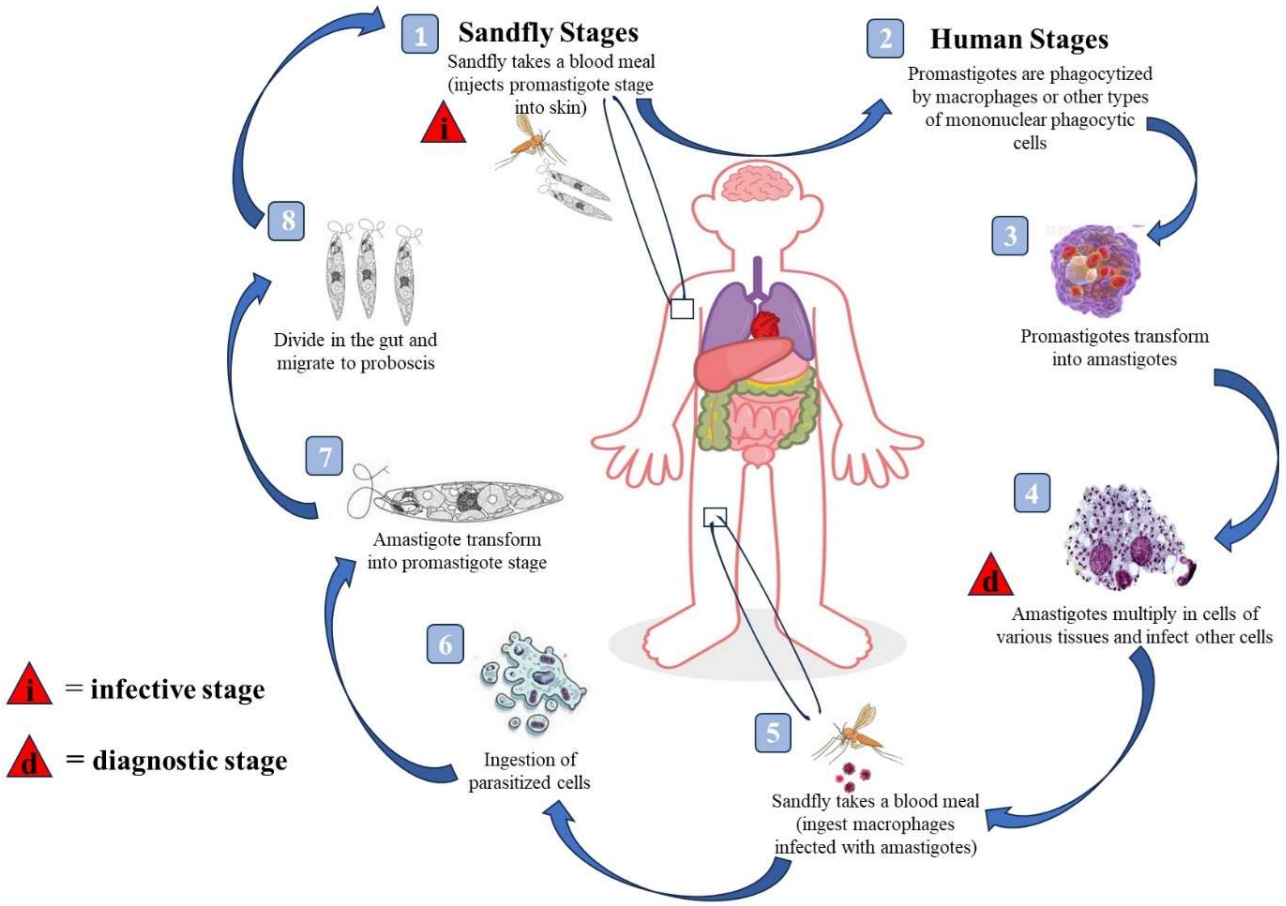

Fig. 2. Life cycle of Leishmania donovani parasite.

PROMASTIGOTES-BASED PHENOTYPIC SCREENING ASSAYS

Potential antileishmanial agents have been screened using leishmania promastigotes cultivated in basic medium as the test parasite. The promastigotes-based assay's widespread use can be attributed to its ease of use and potential for automation. The promastigotes are cultivated *in vivo* at 21–27 degrees Celsius in a medium. The test medications and chemicals are administered to the promastigotes *in vivo* for 48 to 72 hours. Prokaryotic growth is assessed using various cell viability markers, such as MTT and AlamarBlue [25]. According to Gupta and Nishi (2011), the method is straightforward and suitable for high-throughput screening [23]. Promastigotes have different metabolisms and physiological requirements for growth and culture than amastigote (the parasite's host stage). The results of *in vivo* promastigote screening may not accurately represent activity against intracellular amastigotes due to these metabolic and physiological variations [26]. Amastigotes in human hosts grow at 37°C, whereas promastigotes grow at a lower temperature (21-27°C). While promastigotes grow at a pH of neutral, intracellular amastigotes proliferate inside the macrophage phagolysosomes, where they are exposed to the acidic phagolysosomal vacuole environment [27]. Because of these issues, screening against intracellular amastigotes is more valuable than using promastigotes culture for *in vivo* antileishmanial screening.

AXENIC AMASTIGOTES-BASED PHENOTYPIC SCREENING ASSAYS

According to Zakai et al. axenic amastigotes are leishmania cells in amastigote forms that have evolved to thrive in the

medium *in vitro* by offering the ideal temperature of 30°C and an acidic pH of 5.5 [28]. Axenic amastigotes cultivation *in vitro* screening offers a number of benefits. The amastigotes, or parasite host stage, are the target of the test. *In vitro* culture of the axenic amastigotes is simple. Simple cellular growth indicators can be used for the axenic amastigote growth analysis [29]. Screening using axenic amastigotes is easy and affordable. The Axenic amastigotes drug screening technology has been utilized in the past [30]. The viability of cell populations using a 3-(4-5) dimethyl-thiazol–2–yl)–2, diphenyl tetrazolium bromide, thiazole blue (MTT) based method [31], calculating ornithine decarboxylase activity, or using a fluorescent dye like propidium iodide (PI) and fluorescence-activated-cell-sorter (FACS) [32]. A number of Leishmania parasites that express reporter genes have been chosen, and their potential for application in axenic amastigote medication screening has been evaluated. Luciferase-expressing DNA converted Axenic L. baby amastigotes and demonstrated its usefulness in high throughput screening for novel antileishmanial medications [33]. Recently, Shimony and Jaffe (2008) conducted a fast fluorescence assay with Alamar Blue to evaluate medicines on axenic amastigotes of *L. donovani* and *L. tropica* [34]. Nevertheless, the assay is only semi-predictive; it does not assess the compound's ability to enter the host cell or its activity in the unique macrophage phagolysosome environment [23]. The metabolic mechanisms of intracellular and axenic amastigotes may differ. There is a possibility that chemicals may precipitate at an acidic pH because this parasite develops in a medium with a pH of 5.5. The pathophysiological conditions in these tests differ from those in the human host. Therefore, any substances that are active here might not be active in the internalized amastigote form of human macrophage cells.

INTRACELLULAR AMASTIGOTES-BASED PHENOTYPIC SCREENING ASSAYS

The pathophysiology of leishmaniasis sickness is represented by amastigotes forming inside the phagolysosomal vacuoles of macrophage cells [35]. The actual target of the antileishmanial medication candidate is these intracellular amastigotes. Compound libraries must be screened against the proliferation of amastigotes in host cells in order to find new antileishmanial therapeutic candidates. Clinical and laboratory isolates of leishmania cells can also be tested for infectivity using the intracellular amastigotes-macrophage phenotypic assays. Compared to the straightforward promastigotes/axenic amastigotes assays, these assays present a number of difficulties, including (a) choosing the right host cells, (b) individual differences in host cell populations, (c) variable pathogen-cell infectivity and host-cell susceptibility, (d) maintaining the host-parasite cell cultures over an extended period of time, and (e) quantitative and qualitative monitoring of the parasites' intracellular growth. These issues have been resolved recently by developments in transgenic parasite cells with stable constitutive expression of reporter genes, a range of primary and culture-derived host cells, high-throughput sensitive/quantitative cell imaging technologies, and *in vivo* cell culture techniques.

THE HOST CELLS

When leishmaniasis strikes mammalian hosts, macrophages are the main host target cells. Host macrophage cells are necessary for the assay of intracellular amastigotes because they can become infected with leishmania cells and aid in the creation, growth, and proliferation of intracellular amastigotes [35]. There are several ways to collect primary macrophage cells, including peripheral blood monocyte cells (PBMC), bone marrow derived macrophages (BMM), and peritoneal exudate cells (PEC) [36]. PECs are obtained by intraperitoneal injection of 3.0 ml of 3% thioglycollate medium into mice. Five milliliters of ice-cold phosphate buffer saline (PBS) were used to extract the peritoneal exudate cells following three days of thioglycollate medium injection. Heparinized blood is fractionated using Ficoll-Hypaque to produce PBMC, which are then rinsed with Hank's balanced salt solution (HBSS-Sigma H6136) and re-suspended in warm RPMI-1640 medium. By washing with RPMI, BMM is extracted from the mouse's tibia and femur [36]. Although these cell preparations may not have uniform cell populations, the differentiated primary macrophages obtained from multiple sources, including mice and rats, are non-

dividing in nature. Acute promyelocytic leukemia cell line HL-60 [37], pro-monocytic, human myeloid leukemia cell line U937 [38], and human monocytic leukemia cell line THP1 are among the human macrophage cell lines that are accessible. Several mouse macrophage cell lines are available, including RAW 264.7 cells and J774 cells. The macrophage cell population in the cultivated cell lines is uniform. Assays that use dividing host cells must, however, take into account the confusing effects of drug activity on the number of parasites and host cells. Differentiated THP1 cells from several monocyte cell lines produce a non-dividing monolayer similar to primary cells, possess all the traits of macrophage cells, and present a compelling substitute for primary isolated macrophages.

THE PARASITE CELLS FOR INFECTION OF THE HOST CELLS

The genus Leishmania contains about 30 species. However, *L. major*, *L. tropica*, *L. mexicana*, and *L. brazilensis* are utilized for cutaneous leishmaniasis (CL) drug screening, while *L. donovani* or *L. baby* employed for visceral leishmaniasis [23]. Using macrophage cell lines and other primary macrophage cells, the L. infantum parasite has been employed for promastigote and intracellular amastigote screening, specifically for visceral leishmaniasis [36]. L. infantum has been used in a number of *in vivo* investigations in dogs, golden hamster hosts, and mice. Similarly, utilizing macrophage cell lines and other primary macrophage cells, the parasite *L. donovani* has been employed for both intracellular amastigote screening and promastigote screening [38]. *L. donovani* has been used in a number of *in vivo* investigations in dogs, golden hamster hosts, and mice. The parasite's infectious stage is represented by metacyclic promastigotes. The metacyclic stage is acquired by the parasite during the *in vivo* culture's stationary phase.

METHODS FOR INTRACELLULAR AMASTIGOTES GROWTH ANALYSIS

Assays for intracellular amastigotes are intricate procedures that need the cells of leishmania parasites as well as host cells (macrophages). For screening, a variety of macrophage types might be used as host cells (Section 1.2.3.1.). Macrophage cells were planted in plates and kept in an incubator with 5% CO2 at 37°C for the entire night. To differentiate into complete macrophage cells, certain monocytic cell lines (THP1cells) need to be treated with phorbol myristate acetate (PMA). Metacyclic leishmania promastigotes parasites are permitted to infect macrophage cells after they have adhered to the plate surface. Leishmania parasites can infect distinct macrophage cells in different ways. To achieve optimal infectivity, the ratio of macrophages to parasites (multiplicity of infection) must be rigorously standardized. Test chemicals are applied to the infected macrophage cells for 48–96 hours. One of the following techniques is used to evaluate the proliferation of intracellular amastigotes following the treatment of test substances.

MICROSCOPIC METHOD

This method entails introducing leishmania promastigotes or amastigotes into the host macrophage cells. Nucleic acid stains, such as Giemsa and SYBR Green I, are used to stain the infected macrophage cells. Both the test compounds and the conventional medications are used to treat the infected macrophages. Either comparing the percentage of infected cells or the ratio of certain amastigote nuclei to macrophage cell nuclei is how the anti-leishmanial activity of the test medications or chemicals is determined. Traditional microscopic analysis that relies on direct cell and parasite counting by hand is time-consuming. This assay's usefulness is limited by its lack of automation. Digital image-analysis techniques have replaced the traditional microscopic techniques. These techniques use a microscope to take pictures of infected macrophage cells labelled with nucleic acid, and ImageJ software is used to count the nuclei of macrophage cells and amastigote cells [23].

IMAGE-BASED MICROSCOPIC HIGH CONTENT SCREENING

Since these approaches have a high throughput screening capacity and provide crucial information on the infectious

pathogen as well as the target host cell and organ, microscopic imaging-based technologies are effective tools for both primary and secondary screening of therapeutic compounds. Therefore, there is a lot of opportunity for developing a more biology-driven environment for chemical discovery *in vivo* and *in vivo* research using imaging-based high content screening approaches. Using Draq5 nuclear staining, a high content analysis for the wild-type of *L. donovani* has been created. This approach entails calculating the Voronoi diagram and doing a thorough analysis utilizing Image Mining (IM) software. Using the intravital microscopy imaging (IVM) technique with confocal microscopy and Hoechst staining, another high content method was created for *L. donovani*.

FLOW CYTOMETRIC ASSAYS

Moreover, flow cytometry has been used to analyze the leishmania-infected macrophages using fluorescent dyes and monoclonal antibodies. A U-937 host cell model has been used to test the ability of BCECF-Am (29,79bis-(2-carboxyethyl) 5-(and-6)carboxyfluorescein, acetoxymethyl ester), SYTO 17, PKH2-GL (PKH2 Green Fluorescent General cell linker), propidium iodide, and acridine orange to monitor leishmania infection [38]. Longer periods of infection monitoring were not possible with this approach. This method is not ideal for intracellular screening since the infected macrophage cells must be incubated with the test chemicals or medicines for two to four days [26]. Flow-cytometry analysis of infected macrophages has been performed using a number of Leishmania-specific monoclonal antibodies. For investigation based on flow cytometry, a particular monoclonal antibody against the leishmania lipophosphoglycan (LPG) has been employed [39]. Accurate evaluation of the activity of test and standard drugs against intracellular amastigote was made possible by these antibody-based techniques. Nevertheless, the antibody-based immunostaining techniques are expensive and time-consuming for extensive screening. The use of antibody-immunostaining-based techniques for extensive high-throughput screening initiatives may be restricted. This method might not distinguish between intracellular amastigotes that are alive and those that are dead [26, 39]. In these tests, drugs that have cytostatic effects on leishmania amastigotes might not be active.

TRANSGENIC LEISHMANIA CELLS WITH REPORTER GENES

Reporter genes are externally inserted genes that are expressed into clearly quantifiable phenotypic proteins that are easily distinguished from endogenous protein backgrounds. The introduction of these exogenous genes makes it easier to measure the population expansion of transgenic cells or microorganisms and can be used to search for antimicrobial drugs. Transgenic cells with a reporter gene can be produced by either integrating the reporter gene on a specific location of the target cells' genome, usually the rDNA locus, or by episomally transfecting recombinant plasmids with a copy of the reporter gene. Fluorescent protein reporter genes include infra-red fluorescent, mCherry protein, and green fluorescent protein (GFP). Other reporter genes that were created with various enzymes include β-galactosidase, β-lactamase, and Luciferase [23]. Since the protein is naturally luminous, transgenic cells with fluorescent reporter genes do not need cofactors or substrates, giving them an advantage over catalytic reporter genes.

Green Fluorescent Protein (GFP)

The fluorescent protein known as GFP comes from the jellyfish, Aequorea victoria. Compared to traditional non-reporter gene assays, GFP-based assays are simpler, provide time-dependent growth monitoring, are less expensive, and have improved biosafety *in vivo* animal models. For drug screening experiments, transgenic Leishmania spp. cells expressing GFP have been created via stable transfection or episomal transfection [40]. The lack of GFP fluorescence protein expression in the transgenic GFP leishmania cells may make them insufficient for spectrofluorometric reader measurement. For tests involving fluorescence measurements, the GFP transgenic leishmania cells might not be appropriate. To boost the fluorescence intensity in transgenic parasites, a multimeric version of GFP was created and produced in Leishmania

promastigotes. The GFP reporter gene was incorporated into the parasite's genome downstream of the 18 S rRNA gene promoters to create a stable transfected leishmania parasite [40].

Red Fluorescent Protein (RFP)

Although GFP is quite stable and produces a lot of fluorescence, the light wavelength needed to excite it is near the ultraviolet. Leishmania cells may sustain damage and lose some of their viability if exposed to this light. On the other hand, the safe area is where the excitation maxima of parasites with red fluorescent labels are located. A transgenic strain of *L. major* that expresses Red Fluorescent Protein 1 (RFP) has been developed *in vivo* in a mouse model and has been useful in identifying the location of sandfly bites [41]. RFP-expressing transgenic *L. infantum* cells have been used to document neutrophil recruitment and their function in non-ulcerative leishmaniasis [42]. A transgenic leishmania parasite that expresses mCherry, a modified version of RFP, has recently been created and used for screening both *in vivo* and *in vivo* [43]. The fluorescent DSRED2 molecule was chromosomally integrated to create a transgenic cell line of *L. amazonensis*. Using homogeneous populations of primary mouse macrophages harboring transgenic fluorescent DSRED2 *L. amazonensis* amastigotes, a High Content Analysis test was created. The parasite load of fluorescent DSRED2 amastigotes-hosting macrophage cultures was monitored with the aid of this multi-parametric test [44].

Infrared Fluorescent Protein (IFP)

According to Shcherbo et al. tissue penetration is poor for fluorescence with an emission spectrum in the visible region [45]. From the bacteriophytochrome of Deinococcus radiodurans, a novel fluorescent protein with a near-infrared emission spectrum has been identified [46]. Compared to fluorescence reporter proteins, infrared fluorescent proteins (iRFPs) have a greater ability to penetrate tissue with their light. This protein has a near-infrared emission spectrum, and there is no background interference from organs or tissues in the fluorescence that is released [47]. Adenovirus serotype 5 *in vivo* infection of the mouse liver has been effectively imaged using fluorescent iRFPs [48]. The photosynthetic bacteria Rhodopseudomonas palustris produces a novel infrared fluorescent protein (iRFP) that has recently been modified to have a greater penetrating capacity (Ref). The IFP 1.4 and iRFP reporter genes are consistently overexpressed in two strains of L. infantum that were recently reported to have been generated [48]. The transgenic *L. infantum* cells' bio-photonic characteristics were contrasted during the promastigote and amastigote phases. To enhance the fluorescence emission of the chosen reporter in intracellular amastigotes, the transfection constructs were modified to incorporate the regulatory sequences of the genes A2, AMASTIN, and HSP70 II, which are differently expressed in leishmania amastigotes. In leishmania amastigotes, the transgenic leishmania strain that bears the iRFP gene under the regulation of the *L. infantum* HSP70 II downstream region (DSR) demonstrated strong and specific production of iRFP. It was also reported that this strain was used for phenotypic screening with *ex vivo* splenocytes from BALB/c mice infected with infrared [48].

β-GALACTOSIDASE

The enzyme β-galactosidase hydrolyzes the colorless substrate o-nitrophenyl-β D-galactopyranoside (ONPG) to produce the yellow o-nitrophenol (ONP). Drug screening techniques were conducted using transgenic Leishmania promastigote that expressed β-galactosidase [49]. Colorimetric detection is a benefit of B-galactosidase. However, the use of these transgenic parasites for general drug screening purposes, especially for intracellular amastigote screening and *in vivo* screening, is hindered by some common drawbacks, such as their large size (the monomer is 116 kDa), costly substrate, and the endogenous expression of β-galactosidase by some mammalian cell types, including macrophages [50, 51].

β–LACTAMASE

An enzyme called β-lactamase hydrolyzes the colorless substrate nitrocefin to produce a colorful product called a chromogenic cephalosporin. At 490 nm, this product can be tested colorimetrically. β-galactosidase is a larger protein than β-lactamase. The β-lactamase reporter gene was introduced into two transgenic leishmania parasite cell lines, *L. major* and *L. amazonensis* [51]. Through stable transfection, the β-lactamase gene was incorporated into the ribosomal RNA (rRNA) region of the genome, enabling high-level, stable expression of the

enzyme and creating transgenic leishmania cells. Transgenic intracellular amastigotes were utilized to test the efficacy of a few common antileishmanial medications, showing that this technique might be applied to phenotypic drug screening processes [52].

LUCIFERASE

Fireflies contain an enzyme called luciferase, which catalyzes the transformation of luciferin into oxyluciferin, which results in luminescence. Because oxyluciferin, the reaction result, is still in an electrically excited state, luminescence is generated. As oxyluciferin returns to its ground state, a photon of light is released by the reaction. Luciferase-expressing cell lines of several transgenic leishmania parasites (*L. donovani*, *L. infantum*, and *L. major*) have been created [53]. Two significant benefits of luciferase assays are their great sensitivity and the lack of background activity in the host cell. Using imaging analysis, a transgenic cell line of L. amazonensis that expresses firefly luciferase has recently been utilized to track leishmania infection in real time. Since the vitality of both parasites within the host cells is monitored, the advantage of this technology depends on the ability to conduct studies on live cells, which speeds up and improves the accuracy of the study [26].

TRANSGENIC LEISHMANIA CELL MODELS: ADVANTAGES AND DISADVANTAGES FOR PHENOTYPIC SCREENING

New antileishmania drug development and phenotypic screening have benefited greatly from the transgenic leishmania cell lines that exhibit constitutive and stable expression of reporter proteins. Large-scale, high-throughput, high-content screenings have made substantial use of these models [44]. Depending on the type of reporter gene, the intracellular transgenic parasite cells can be observed via chemiluminescence readers, flow-cytometry, or digital image analysis. Drug resistance markers must be used to select transgenic cells in order to create transgenic leishmania models. To maintain the reporter genes' episomal expression, the transgenic cells were cultured under the selection drug's contact pressure. According to Buckner and Wilson (2005), if the reporter gene is expressed, the number of copies of the transfected plasmid may determine the relative expression of the reporter protein rather than the drug's activity [50]. Once the integration of the reporter gene into the leishmanian genome and the stable expression of the reporter protein are verified, the use of a selection medication may not be required in cases of stable transfections and genetic integration. For large-scale screening, the assays based on transgenic parasites with luciferase reporter genes are costly since they require cell lysis buffer and substrate [48]. Table 2 lists the growth analysis techniques for intracellular amastigotes along with their benefits and drawbacks.

Table 2: The growth analysis techniques for intracellular amastigotes.

Type of Assay	Method	Advantages	Disadvantages
Classical microscopic evaluation	Nuclear staining of infected macrophage cells	Gold Standard Reliable	Labor intensive, Time consuming, Low Throughput, Human Bias
Digital-Image analysis based assays	Analysis of digital images of the intracellular leishmania amastigotes	Automated, Multi-parametric High throughput Screening, High-content analysis	Costly instrumentation Limited facility
Flow cytometric assays	Antibodies-based analysis	Specific, Less background signal	Sensitivity issues
Transgenic leishmania-Reporter gene assays	Transfections of reporter gene	Live imaging Live growth analysis	Require drug selection for episomal expression. Relative expression of the reporter gene may depend on the copy number of the transfected plasmid. Altered physiological properties of the parasite

IN VIVO ASSAYS FOR ANTILEISHMANIAL SCREENING

For *in vivo* anti-leishmanial research, animals that exhibit pathological reactions comparable to those seen in humans following leishmania parasite infection may be chosen. The liver and spleen are deep organs where visceral leishmania parasites live. Therefore, parasites must be able to enter macrophage cells, change into amastigotes there, and live in deep organs like the liver and spleen in animal models of visceral leishmaniasis. Numerous animal models are available for studying visceral leishmaniasis, including etiology, prophylaxis, metabolic alterations, host-parasite interactions, parasite maintenance, and the assessment of drug leads' antileishmanial activity. Intravenous parasite inoculation, chemical dosage, making impression smears of weighted livers, staining slides, and examining parasitemia and other host-parasite interactions are all examples of these *in vivo* experiments [23]. There are a number of animal models that have been developed, each with pros and cons, but none of them can replicate the human experience of visceral leishmaniasis. A number of animals were created as VL experimental hosts, including dogs, Syrian golden hamsters, BALB/c mice, and monkeys (Indian languor, squirrel, and vervet). A good animal host for the leishmania parasite is crucial for various research, such as host-parasite interactions, immunological alterations, pathogenesis, biochemical alterations, and prophylaxis, in addition to assessing treatment activity. Along with other important data such as bioavailability, pharmacokinetic and maximum tolerated dose, animal models aid in the evaluation of antileishmanial activity and aid in the determination of the route, treatment dose, duration of dosing, therapeutic window, and toxicity of novel leishmanicidal leads. A medication should ideally be delivered orally, have an intrusive method of delivery, be efficacious at low doses with a short half-life (about a week), and not be hazardous even at greater dosages.

RODENT MODELS

Several mouse models were used for antileishmanial medication screening because they are the favoured choice for primary *in vivo* investigations. In comparison, rodents are tiny. As a result, they are less expensive, take up less space and maintenance, use less testing chemical, and are simple to handle during investigations. Syrian golden hamsters and BALB/c mice are common rodent models for visceral leishmaniasis [54]. The pharmacological effects of anti-leishmanial effectiveness, toxicity profiling, host-pathogen interaction, and disease etiology have all been assessed using these rodent models of leishmaniasis. These are offered as inbred strains that aid in producing less variable and repeatable results. Mice are vulnerable to many Leishmania parasite species. For *L. donovani* susceptible, resistant, and intermediate strains, inbred mouse strains have been extensively employed [55]. To make sure that the test chemicals are evaluated for their effects in a suitable manner, the Leishmania parasite infection in several mouse strains must be described. Mice with the Foxn1nu mutation in athymic mice and the Prkdcscid mutation in SCID mice offer a paradigm for treating VL in immunocompromised patients [56].

MICE MODEL

When the amastigote or metacyclic promastigote inoculum is given intravenously, the BALB/c mouse is a strain that is most frequently found to have repeatable levels of infection (parasitemia). All BALB/c mice were first split up into groups of five, and each group received an intravenous infection with the parasite *L. donovani*. At day 7 of post-infection, the test chemicals are produced in various concentrations and administered to mice intraperitoneally, intravenously, or orally for five days in a row (day 7 to day 11). At the fourteenth day after infection, all infected mice are killed. All mice had their liver and spleen removed and weighed. Mice from every group are monitored for changes in weight and behavior during the whole experiment. On slides, impression smears are taken from each mouse's liver and spleen. Giemsa is used to stain smear-containing slides after they have been fixed with methanol. With the aid of a microscope, the total number of leishmania amastigotes per 500 liver cells is determined. Liver smears of untreated and treated mice are examined to determine the parasitemia of each mouse [23].

RAT MODEL

The African white-tailed rat, *Mastomys albicandatus*, has also been studied for long-term studies with *L. donovani* and *L. Brazilians*, as well as *in vivo* maintenance [57]. Another excellent susceptible animal host for *L. donovani* is the cotton rat, or Sigmodon hispidus [58]. Following the onset of the first clinical symptoms, the Leishmania infection persisted in this host for three to four months. The experimental model for visceral leishmaniasis utilizing *L. donovani* as a parasite was also developed using *Mastomys natalensis* (Natal multi-mammate Rat) [59]. Rat animal models are subjected to same experimental protocols as mice. In this case, rats are a better model for long-term research since they can maintain the parasite for longer than mice [23].

HAMSTER MODEL

Similar to human VL, the Syrian golden hamster, *Mesocricetus auratus*, has developed a suitable model for the disease. It causes a synchronous infection in the liver and spleen that can progress into a chronic, incurable illness [60]. It mimics the histological and clinical characteristics of human disease, including hyper-gammaglobulinemia, large splenomegaly, cachexia, a progressive increase in the visceral parasite burden, and finally mortality [60]. Although the majority of VL research uses intravenous, intracardiac, or intraperitoneal inoculations of rodents with 106–108 parasites [61], VL has also been produced by intradermal inoculation, which is thought to be a much more natural infection method. By allowing the parasites to spread naturally through vector sand fly bites, a novel hamster model of progressive leishmaniasis was created more recently. Monitoring parasites in the spleen, liver, and bone marrow using limiting dilution cultures or by imprinted smears with splenic biopsies are two methods used to detect parasitemia in tissues [62]. The hamster has been used as a model for VL in a number of approaches. Traditionally, a splenic biopsy that aids in carrying the same experimental animal's identical parasitic load was used to determine the pre-treatment parasitic burden. The incapacity of this approach to evaluate the delayed action of medications is one of its drawbacks.

In order to evaluate the long-acting and delayed action medicine in the model, this technique was then adapted to perform repeated spleen biopsies on the same animal at various intervals of days 7, 14, and 28 [23]. The benefit is that all antileishmanials are effective against liver and spleen parasites, and biopsy can be used to track infection status both before and after treatment. The best animal model for studying VL is the golden hamster, which mimics the disease's etiology and clinical manifestations in both humans and canines [54]. With the use of virulent transgenic *L. donovani* that consistently express a reporter luciferase, the degree of infection was recently determined by real-time imaging of a golden hamster model. This was contrasted with real-time RT-PCR to quantify both Leishmania and host transcripts [63].

REAL-TIME IMAGING OF A MURINE LEISHMANIASIS MODEL

In vivo real-time fluorescence imaging is now possible to examine the time-dependent course of Leishmania infection in parasitized tissues thanks to episomally transfected Leishmania parasite strains with increased green fluorescent protein and stably transfected infrared fluorescent Leishmania parasite strains. The amount of fluorescence showed the real-time effectiveness of a test drug or vaccination and was connected with the quantity of Leishmania parasites in the tissue. This method has a number of benefits over the traditional microscopic-based method. Here, it was possible to analyze the change in infection level in real time without causing the animal any pain. These include increased sensitivity and the capacity to obtain real-time information on the infection's development and dissemination.

DOG MODEL

In the Mediterranean region, the Middle East, Asia, and Latin America, wild dogs and dogs are the primary carriers of zoonotic visceral leishmaniasis, which is caused by *L. infantum*. There is now more interest in using dogs as an animal model for visceral leishmaniasis because they are the primary reservoir of the disease [64]. Visceral leishmaniasis in dogs is a multi-systemic illness with a range of clinical manifestations. Using *L. donovani*, *L. infantum*, or *L. chagasi*, dogs were created as an experimental model for Leishmania

infections in the 1990s. Due to their ability to replicate the natural infection 32 in a manner comparable to that of humans, dogs are valuable laboratory models [65]. Although some researchers claim a high success rate with mixed breeds, German shepherd dogs are said to do better than beagles. Liver or spleen biopsies are used to determine the degree of infection [66].

NON-HUMAN PRIMATE (NHP) MODEL

Due to a number of physiological and anatomical variations, certain observations made in rat models are not applicable to human hosts. Nonetheless, the morphology, immunology, and physiology of non-human primates are comparable to those of humans. For biomedical research, they are therefore useful primate models. These are pricy lab animals that are challenging to acquire and care for. Additionally, for ethical considerations, the institutional animal committee prohibits the use of monkeys in biomedical research. As a result, very few labs around the world are developing primate models, particularly for diseases like visceral leishmaniasis that are often ignored.

A non-human primate model of visceral leishmaniasis would make it easier to research many facets of the illness and hasten the creation of vaccines and the evaluation of novel therapeutic alternatives. Final preclinical trials for the safety and effectiveness of any new drug candidates or vaccinations are typically conducted on monkeys [23]. Owls and squirrel monkeys have undergone anti-leishmanial screening. *Aotus trivirgatus* (owl monkeys) and *Saimiri sciureus* (squirrel monkeys) both acquired an acute and fulminated, but brief, illness, according to the initial attempt to infect both New and Old World monkeys [67, 68]. The species was highly susceptible to a single intravenous inoculation of 33 hamster-spleen-derived *L. donovani amastigotes,* which consistently produced a progressive acute fatal infection that resulted in death between 110 and 150 days after infection, according to attempts to establish VL in Presbytis entellus (Gray langur). All of the immunopathological clinic characteristics seen in human VL were present in the infected animals. Additionally, preclinical testing of possible antileishmanial medications and vaccines has been conducted using the Indian language [23].

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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