Identification of Anti-Leishmanial Leads from Open Access Pathogen Box

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School of Graduate Studies

This is to certify that the thesis prepared by Markos Tadele, entitled *"Identification of Anti-Leishmanial Leads from Open Access Pathogen Box"* and submitted in partial fulfillment of the requirements for the Degree of Master of Sciences in Pharmacology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

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Addis Ababa University, 2018

Leishmaniasis is a vector-borne disease caused by an obligate intracellular protozoan of the genus Leishmania. The ranges of drugs available to treat this disease are limited; therefore, there is a substantial need to develop new medicines or drug combinations. Aiming to find potential anti-leishmanial leads, we screened MMV Pathogen Box for two strains of leishmania parasites. In this optimised, medium throughput primary screening assay, all 400 compounds were screened against promastigotes and amastigotes stages of *L. donovani* and *L. aethiopica.* The screen yielded a total of 16 hits with IC_{50} ranges from 0.01 to 0.555 µM on anti-promastigote assay and 0.05 to 0.7 µM on intracellular amastigote assay, respectively. Two compounds, known by MMV690102 and MMV688262 were identified as lead compounds for *L. aethiopica* and *L. donovani*. Cytotoxic effect of selected hits and synergistic effect of lead compounds with common reference drugs was also investigated. All selected compounds demonstrated good safety for the mammalian cell they were tested. The anti-TB drug (delamanid) showed synergistic effect with Amphoterecin B. This indicates the prospect of this compound for combination therapy. MMV690102 demonstrated inhibitory activity on both tested strains indicating its broad spectrum in activity. Future works should investigate antiamastigotes activity of those ‘hits’, which are not covered in the present study, and Identified lead compounds should progressed to other pre-clinical studies and their mechanism of action should be investigated using target based experiments.

KEYWORDS: ***Leishmania donovani, Lishmania aethiopica*, promastigotes, Intracellular Amastigotes, MMV Pathogen Box, drug screening, *in vitro***

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Acronyms and Abbreviation

AB :	Amphotericin B Deoxycholate
AHRI:	Armauer Hansen Research Institute
BCG :	Bacillus Calmette Guérin
CC_{50}:	Cytotoxic Concentrations that kills 50% of the cells
LCL/CL :	Localized Cutaneous Leishmaniasis/Cutaneous Leishmaniasis
DCL :	Diffuse Cutaneous Leishmaniasis
Ddn:	Deazaflavin-dependant nitroreductase
DHFR:	Cryptosporidium dihydrofolate reductase
DMSO:	Dimethyl Sulfoxide
EC_{50}/IC_{50}:	Effective Concentration which inhibits 50% of the cells
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
HBC :	High Burden Countries
HEK293:	Human Embryonic Kidney cells
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HepG2:	Human liver carcinoma cells
HINBCS :	Heat Inactivated New Born Calf Serum
HIV :	Human Immunodeficiency Virus
IR:	Infection Rate
KA:	Kala-azar
L-AB :	Liposomal Amphotericin B deoxycholate
LRDL:	Leishmaniasis Research and Diagnostic Laboratory
M199:	Medium 199
MCL:	Mucocutaneous Leishmaniasis
MF:	Miltefosine
MIC :	Minimum Inhibitory Concentration
MMV:	Medicine for Malaria Venture
MoH :	Ministry of Health
MRC5:	Human lung fibroblast cells
NCBI:	National Centre for Biotechnology Information
NTR:	NADH-dependent bacterial-like nitroreductase

PBS:	Phosphate-Buffered Saline
PKDL:	Post Kala-azar Dermal Leishmaniasis
PM:	Paromomycin
PMM:	Peritoneal maurine macrophages
PV:	Parasitophorous Vacuoles
RPMI 1640:	Roswell Park Memorial Institute 1640 Medium
SbV and Sb:	Pentavalent Antimony
SI:	Selectivity index
SNNPR:	Southern Nations, Nationalities, and Peoples' Region
SSG:	Sodium Stibogluconate
THP-1:	human leukemia monocyte THP-1 cells
VL:	Visceral Leishmaniasis
WHO:	World Health Organization

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1. Introduction

1.1. Overview of leishmaniasis

Leishmaniasis is a vector-borne disease caused by an obligate intracellular protozoan of the genus Leishmania. It broadly manifests as Localized Cutaneous Leishmaniasis (LCL), Diffuse Cutaneous Leishmaniasis (DCL), Mucocutaneous Leishmaniasis (MCL) and visceral Leishmaniasis (VL), which are still responsible for considerable morbidity of a vast number of people in endemic areas (1,2).

1.1.1. Localized Cutaneous Leishmaniasis

LCL is the most common form of leishmaniasis, it is also known as 'Oriental sore'. Lesions primarily appear as typical erythema where an infected sandfly has bitten the host. The erythema developed into papules, then to skin ulcers (Fig. 1A). Lesions are usually formed on exposed areas, such as face, arms and legs and most of them are localized (3). Gradually, the lesion enlarges. LCL lesions vary in size, clinical presentations and time to cure (4) and the incubation period can last from few days to months. In the Old World (regions of North and East Africa, Central Asia, and the Middle East) LCL is usually caused by *Leishmania major, Leishmania tropica,* and *Leishmania aethiopica*. In the New World (i.e. Central and South America, the USA, and Mexico) the species causing the different form of CL are *Leishmania mexicana, Leishmania venezuelensis, Leishmania amazonensis, Leishmania braziliensis, Leishmania panamensis, Leishmania guyanensis and Leishmania peruviana* (4).

1.1.2. Diffuse Cutaneous Leishmaniasis

Under certain conditions, *L. aethiopica* , *L. mexicana* , and *L. amazonensis* can cause a diffuse form of the disease, which is characterized by development of nonulcerative lesions away from the initial site of infection (4). DCL produces disseminated and chronic skin lesions resembling those of lepromatous leprosy (Fig. 1E) and the lesions do not have a tendency to self-cure (5–7).

1.1.3. Mucocutaneous Leishmaniasis

Lymphatic or haematogenous dissemination of parasites to oropharyngeal mucosal sites leads to disfiguring mucosal lesions, called MCL (4). The causative agent of MCL in the Old World is *L. aethiopica* (rare), and *L. braziliensis, L. guyanensis, L. mexicana* , *L. amazonensis* and *L.*

panamensis in the New World (3,4). MCL caused by *L. braziliensis* and *L. Panamensis* is characterized by extensive destruction of naso-oral and pharyngeal cavities with hideous disfiguring lesions (Fig. 1B), mutilation of the face and great suffering for life. MCL is also known as espundia (4). MCL may progress to metastatic lesions on buccal or nasal mucosa following the apparently complete resolution of the initial oriental sore (3,4).

1.1.4. Visceral Leishmaniasis

Human VL caused by *Leishmania donovani* or *Leishmania infantum* is a severe disease with generalized spread of the parasites to reticuloendothelial system, such as spleen, liver and bone marrow (8). VL is the most dreaded and devastating amongst the various forms of Leishmaniasis and if left untreated, it is usually fatal. The disease is also known as *Kala-Azar, Black Sickness, Black Fever, Burdwan fever, Dumdum fever or Sarkari Bimari*. VL is typically caused by the *Leishmania donovani* complex, which includes two species: *Leishmania donovani donovani* and *L. d. infantum* (9). VL is characterized by prolonged fever, splenomegaly, hepatomegaly, substantial weight loss, progressive anemia, pancytopenia, and hyperglobulinaemia. The parasite invades and multiplies within macrophages (free mononuclear phagocytic cells) and affects the reticuloendothelial system including spleen, liver, bone marrow, and lymphoid tissue (9,10)

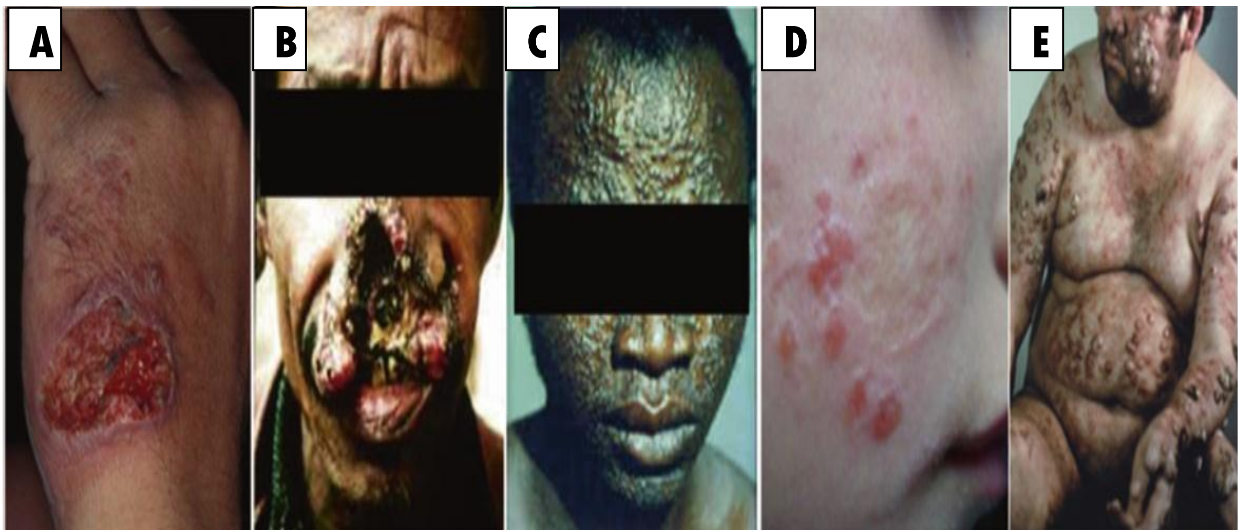

Figure 1: various forms of Leishmaniasis. (A) cutaneous Leishmaniasis lesion on the hand, (B) mucocutaneous lesion of the mouth and nose, (C) facial lesions in a case of post-kala-azar dermal Leishmaniasis, (D) emerging lesions within the old CL scar, (E) lesions throughout in diffuse cutaneous Leishmaniasis (11).

After recovery, patients may develop a chronic CL form called 'post Kala-azar dermal Leishmaniasis' (PKDL) which usually requires a prolonged and expensive treatment (12). The

cause is not known but it is believed to occur as a result of inadequate treatment (13) or poor immunity system in VL patients (11). PKDL presents with a variety of skin lesions such as papules, macules and nodules or a combination of these. The macules are often confused with lesions of vitiligo or leprosy. PKDL may also affect the buccal and genital mucosa and the conjunctiva.

1.2. Etiology, vectors and reservoir hosts of Leishmaniasis

1.2.1. Etiology

The disease is transmitted by various species of intracellular protozoan parasites of the genus Leishmania. The taxonomy of Leishmania is very complex. Classification is often difficult. However, two commonly used classifications are widely accepted. The first classification is based on intra-vectorial development of the parasite. Growth of species of the subgenus Leishmania is restricted to parts of the alimentary tract of the natural vectors anterior to the pylorus at the junction of the midgut and hindgut (suprapylarian development), whereas subgenus Viannia undergoes an additional developmental phase within the hindgut (peripylarian development) (14). The second classification divided the genus by geographic occurrence. Subgenus Leishmania present in both Old world and New World, and Viannia restricted to the New World (14). Leishmania parasites are digenetic organisms with a flagellated promastigotes are found in insect vector and non-flagellated amastigotes are found in mononuclear phagocytic system of vertebrate hosts (15).

1.2.2. Vectors

The sand fly is the vector responsible for transmitting of the disease. Sand flies belong to the subfamily Phlebotominae. Seventy to eighty species are known to transmit the disease (16). Two genera of phlebotomine sand flies are responsible for transmitting Leishmania to humans; species of Phlebotomus sand flies transmit the disease in the Old World and that of Lutzomyia in the New World. Phlebotomine sand flies are widespread in the tropics and subtropics. Wide variations exist among these species in preferred habitats and breeding sites (17). There are more than 30 species of phlebotamus known to transmit the disease, from which *Phlebotomus orientalis*, *Phlebotomus martini*, and *Phlebotomus celiae* have been confirmed to be present in Ethiopia (18,19).

1.2.3. Reservoir hosts

Leishmaniasis is a zoonotic disease with many reservoirs all over the world. It is most commonly found in mice and hamsters. In the Americas, the most common reservoirs are sloths, opossums, small forest rodents, hyrax and peri-domestic dogs (8,20). In the East Africa and India, the most common reservoir hosts are hyraxes and domestic dogs (18,21). Humans are also considered as potential reservoir host since VL in these areas is thought to be anthroponotic (4,18,22). Control and elimination of leishmaniasis has been hampered by the fact that the disease is primarily a zoonosis with large reservoirs of rodents, dogs and other animals (23).

1.3. Morphology and life cycle of the parasites

The parasites exist in two main morphological forms: the amastigotes in vertebrate hosts and promastigotes in invertebrate hosts (24). The promastigote forms are seen in the gut of the sand fly, where the parasite reaches the buccal cavity. Promastigotes are extracellular, elongated, flagellated, motile cells ranging in size from 10–20 µm (Fig. 2a). Whereas amastigotes are intracellular, round to oval, non- flagellated, non-motile and ranges in size from 2 to 5 µm (Fig. 2b) (24). This form resides and multiplies within the phagolysosomes of macrophages in reticuloendothelial system of the vertebrate host (23).

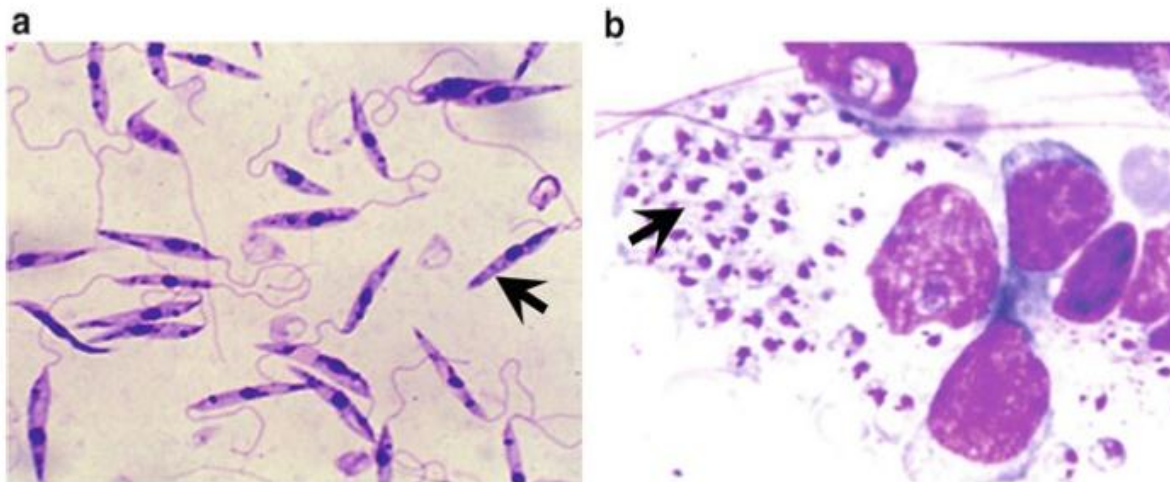

Figure 2: Stages of Leishmania parasite; (a) Extracellular and motile form called promastigotes each bearing a flagellum. (b) Intracellular and non-motile stage called amastigotes (small dots) (23).

1.3.1. Life cycle of the parasites

The disease can be anthroponotic when the cycle involves only the sand fly and human populations, and zoonotic when the cycle involves humans, sand flies and mammalian reservoirs (25). The life cycle starts when the sand fly injects the infective stage (infective promastigotes) from its proboscis during blood meal (Figure 3-1) (16). Following the sandfly bite, some of the flagellates entering the circulation are destroyed, while others enter the cells of the reticuloendothelial system (23). After residing within neutrophils for an as-yet unknown time period after infection, parasites invade macrophages (26). The promastigotes which survive are then phagocytosed by the host's macrophages and other types of mononuclear phagocytic cells where they transform into amastigotes and undergo continuous binary division (Figure 3-4). Multiplication continues until the host cell is packed with the parasites and ruptures, liberating the amastigotes into circulation (23,24). The released amastigotes are taken up by additional macrophages and amastigote multiplication continues with the same fashion. Subsequently, all organs which harbour macrophages especially lymph nodes, liver, spleen and bone marrow are infected (23).

The adult female sandfly unlike the male is a bloodsucker, and when the fly bites an infected human or, in cases of zoonotic disease, an infected mammalian zoonotic reservoir, amastigotes are ingested along with the blood meal (16). The acquired amastigotes are usually from infected phagocytic cells found in the skin or from amastigotes free and in peripheral blood and mononuclear cells in the blood (22).

In the sandfly, amastigotes develop into promastigotes (Figure 3-7), and migrate to the midgut (Leishmania) or the hindgut (Viannia) where they transform into flagellated promastigotes and divide. The extracellular promastigotes undergo further transformation, migration, and binding before some turn to metacyclic-promastigotes, the infective form, which then migrate to the proboscis and the cycle continues when the sandfly injects the promastigotes into the skin of the host during blood meal (16).

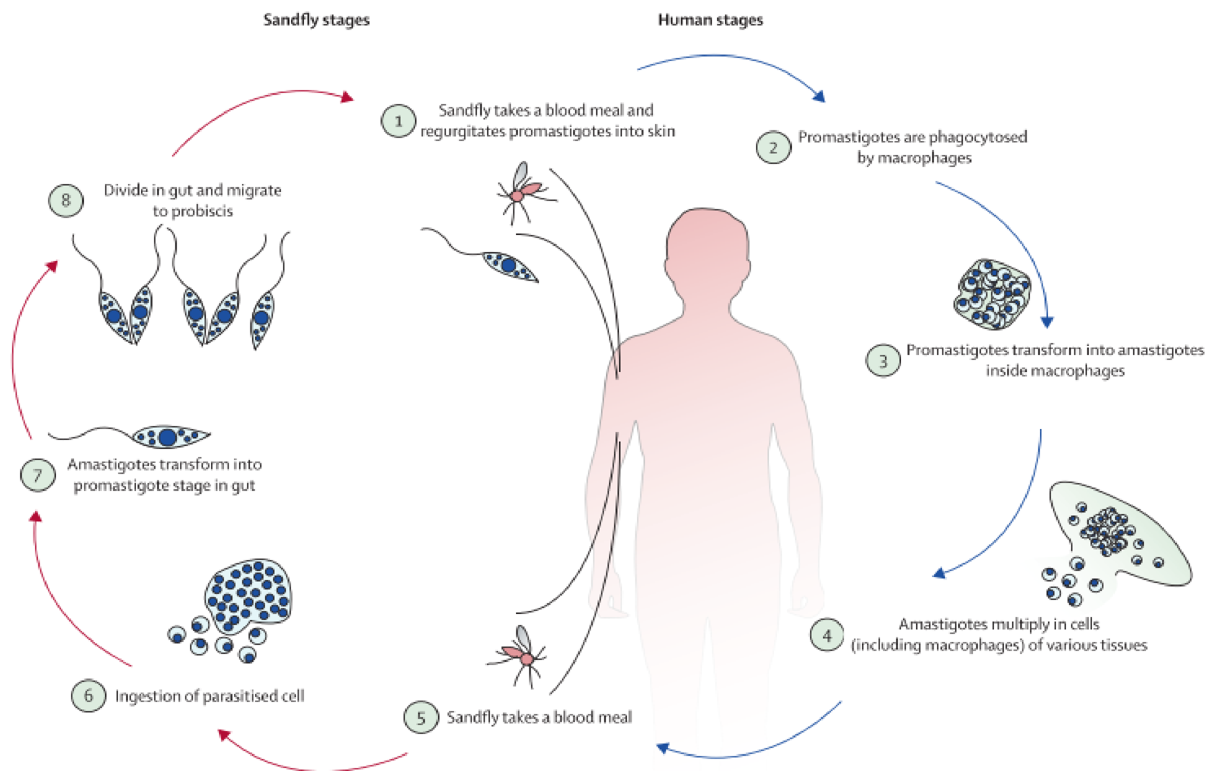

Figure 3: life cycle of Leishmania parasites in the vector and the host (4).

1.4. Distribution of Leishmaniasis

According to the 2017 report of WHO, out of 200 countries and territories reporting to WHO, 97 countries and territories are endemic for leishmaniasis. Among which 65 countries are endemic for both visceral and cutaneous leishmaniasis (27). The global incidence of visceral leishmaniasis decreased from between 200,000 – 400,000 new cases in 2012, to between 50,000 and 90,000 in 2017 (27,28). Published figures indicate an estimated incidence of 2 million new cases per year from which 75% are CL cases. The disease is widely distributed around the world, ranging from inter tropical zones of America, Africa, and extend to temperate regions of South America, southern Europe and Asia (27,29)

The number of cases of leishmaniasis is increasing, mainly because of man-made environmental changes that increase human exposure to the sand fly vector. A slight fluctuation in temperature can allow transmission of the parasite in areas not previously endemic for the disease. Important environmental and climate changes such as alterations in temperature and water storage, irrigation habits, deforestation and global warming can affect the epidemiology of leishmaniasis

and increase the risk (30). Population mobility such as increase travelling to endemic regions, widespread migration from rural to urban areas due to economic reasons and occupational exposure can also increase the risk. Other factors includes poor socio-economic status and low level household, sleeping outside or on the ground, war, dog importation, immunosuppression by HIV or organ transplant, malnutrition and development of drug resistance are also major contributors (28,30–32).

1.4.1. Status of Leishmaniasis in Ethiopia

The Ministry of Health (MoH) estimates the annual burden of VL to be between 4,500 and 5,000 cases. Though the annual burden of CL is not estimated correctly, it is expected to be significantly higher than the annual burden of VL (24). One of the WHO reports listed Ethiopia under top six high burden countries (HBC) (7). A recent WHO report shows the increase of new cases and expansion of disease endemic areas. In recent times, epidemics of CL and VL were observed in Amhara, SNNPR and Tigray regions (7). The number of new cases for CL and VL in these areas is estimated to be 10-50/10,000 people and 1-2.5 cases/10,000 people respectively. The incidence of VL in Tigray region was higher than the two regions i.e. 2.5-10 cases/10,000 people (Fig. 4) (7).

VL has been diagnosed in different parts of Ethiopia. There are known VL-endemic foci in the country (18). The north-western VL focus in Ethiopia covers the Semi- arid Metema and Humera plains in Tigray and Amhara regional states bordering Sudan. The north-western VL focus in Ethiopia has the highest known VL/HIV co-infection rate in the world. Approximately, 30% of VL patients are estimated to have HIV. The other main focus in the southwest occurs in the lower course of the Rift Valley, most notably, the Segen (Aba Roba focus) in Konso woreda, the Weyto valley in the drainage basin of the Chew Bahir Lake, and the Lower Omo plains (18,33).

CL has been diagnosed from many localities in the northern, central, and southern high lands of Ethiopia. Cutaneous form has been extensively studied in the western highlands and lake areas of the Rift Valley. The main areas of transmission include the Ochollo focus in the Rift Valley escarpment above Lake Abaya, the Kutaber area in the eastern Ethiopian plateau near Dessie, the

Aleku area of Wollega zone, the south- west highlands of Bale and Sidamo, and the Sebeta area near Addis Ababa (18,33–35).

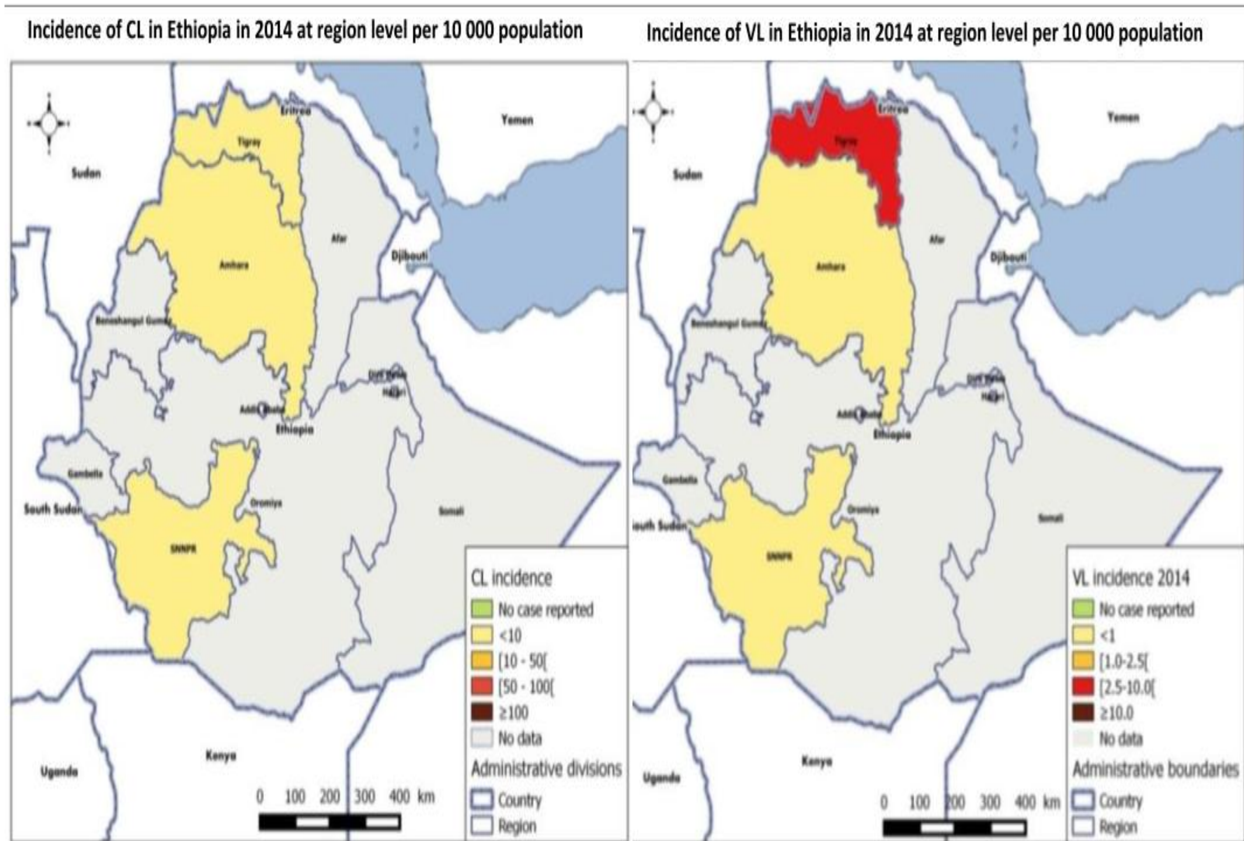

Figure 4: new cases of Cutaneous leishmaniasis (left) and visceral leishmaniasis (right) (7).

1.5. Treatment of Leishmaniasis

There are different ways of therapies for various forms of leishmaniasis and the preferences for first-line and second-line treatments vary based on the type of disease and are often guided by regional practice in relation to what is currently most effective and available (11). Current treatment trends are briefly discussed in the subsequent subsections.

1.5.1. Chemotherapy

1.5.1.1. Pentavalent antimony

An agent of choice for most forms of leishmaniasis was systemic use of pentavalent antimony (SbV), which is present in meglumine antimoniate or sodium stibogluconate (SSG). The precise

mechanism of action of SbV in leishmaniasis is not well understood but is probably multifactorial acting directly on molecular processes of the parasite as well as influencing macrophage parasiticidal activity (36).

1.5.1.2. Amphotericin B

The polyene Amphotericin B is an effective anti-leishmanial agent which works by binding to membrane *ergosterol* causing membrane instability. Both the deoxycholate and the liposomally encapsulated (trade name Ambisome) formulations of Amphotericin B have been used clinically in leishmaniasis (37). Unlike CL, several efforts been made to advance the treatment options for VL; CL has been neglected until recently. But currently, a randomized, double blind, placebo controlled phase 2 clinical trial is undergoing to investigate efficacy of topical liposomal Amphotericin B gel on CL of Leishmania species *L. major* and *L. tropica*. This study is registered at clinicaltrials.gov of the US National Institute of Health with identifier code of NCT02656797. The study strives to bring alternative treatment to CL (38,39).

1.5.1.3. Paromomycin sulphate

Paromomycin (PM) is an aminoglycoside antibiotic which works by blocking protein synthesis via binding to 16S ribosomal RNA. In leishmaniasis, it is used for systemic use (at 15 mg/kg) alone for up to 21 days for Indian VL. Combination of PM with liposomal Amphotericin B or SSG is also used for Indian or East African VL (40). A recent clinical trial indicated that PM can be highly efficacious when used in combination with another antileshmanial drug like SSG. A 17-day SSG and PM combination treatment was found to have a good safety profile and similar efficacy to the standard 30 day SSG treatment. The reduced duration of treatment with the combination compared with SSG (17 versus 30 days) was shown to reduce burden on hospitals and patients and other associated costs (41).

1.5.1.4. Pentamidine

The precise antimicrobial mechanisms of pentamidine are unknown but the drug may interfere with the biosynthesis of macromolecules such as DNA, RNA, phospholipids, and proteins (40).

1.5.1.5. Miltefosine

Originally investigated as an antineoplastic agent, the alkylphosphocholine miltefosine is the only oral agent being used for Indian and East African VL both in children and adults where the

dose is increased weight-based (40). Miltefosine is highly effective oral drug, but it is potentially teratogenic and restricted in some patients, especially in pregnant women. To increase the efficacy and safety of miltefosine an open label, Phase III, randomized, controlled clinical trial with clinical trials registry identifier NCT03129646 is undergoing. The study compares the efficacy and safety of two combination regimens of Miltefosine and Paromomycin with the standard SSG-PM for the treatment of primary adult and children VL patients in Eastern Africa. The study completion is planned for November, 2019 (42,43)

1.5.2. Physical therapy

1.5.2.1. Cryotherapy

Liquid nitrogen application directly to CL lesions either once or multiple times, up to five times, every 3–7 days has been used in the treatment of Old World CL caused by *L.* tropica , *L. aethiopica* , and *L. infantum* or in combination with intra-lesional antimony for *L. major* (44).

1.5.2.2. Heat therapy

Heating lesions at 50 °C for 30 seconds up to three times can be used to hasten resolution of Old and New World CL lesions. The result is comparable to intra-lesional or systemic antimony therapy. This treatment has also been found to be effective against CL in HIV-infected patients who do not respond to antimonial therapy (1). The combination effect of heat therapy with systemic antileishmanial drug miltefosine is under investigation (NCT02687971). The study is currently undergoing to determine the efficacy and safety of a combined therapy using thermotherapy (one session, 50 °C for 30") + miltefosine at a standard dose of 2.5 mg/kg/day for 21 days for the treatment of uncomplicated CL in Peru and Colombia (45,46).

1.5.3. Immunotherapy

Vaccines consisting of heat-killed Leishmania plus BCG (Bacillus Calmette Guérin) or defined recombinant antigens together with Granulocyte-macrophage colony-stimulating factor (GM-CSF) have been used in small numbers of patients with refractory MCL or DCL (47). But the success rate is not still known. For decades, significant research efforts have been made to produce effective vaccines, but none approved for human use. Currently, a phase II vaccine trial known by NCT02894008 is being conducted in PKDL patients. This is by far the most successful vaccine trial. The trial is being done to assess the safety and compare the humoral and

cellular immune responses generated by the candidate vaccine in patients, and observe any clinical changes in the disease over a 42 day period following vaccination (48,49).

1.6. Medicine for Malaria Venture Pathogen Box

The Pathogen Box (PB) contains 400 most promising, free-of-charge pure compounds that are available to researchers to apply to their areas of disease investigation. These compounds are supplied by Medicine for malaria venture (MMV) through its project The “Open Source Pathogen Box” funded by the Bill & Melinda Gates’ Foundation. The box is designed to provide starting points for the discovery of new medicines against a range of Type II and Type III diseases (Fig. 5A). This would be done by providing active compounds in an Open Source mode in collaboration with global partners.

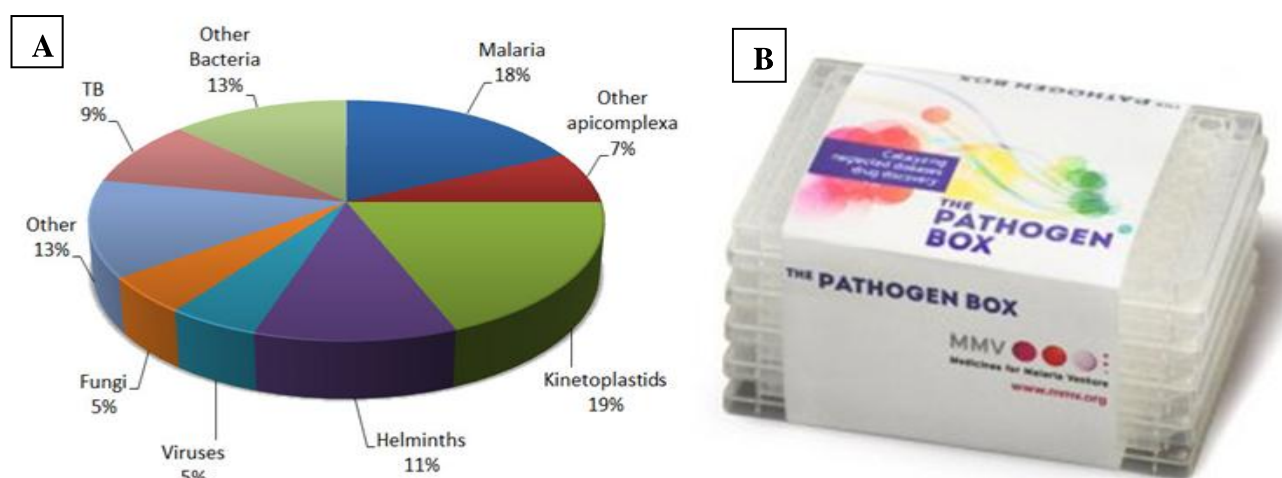

Figure 5: The Pathogen Box: A) compounds classified based on target disease sets, B) sealed plates containing test compounds

MMV Pathogen Box has been screened for different pathogens. Screening of PB compounds on *Trypanosoma brucei brucei* uncover new starting points for anti-trypansomal drug discovery (50). Another study on *Neospora caninum* by Müller et al. (51) identified new compounds with profound activities. A screening made by Hennessey et al (52) come across with 3 new inhibitors with dual efficacy against *Giardia lamblia* and *Cryptosporidium parvum* (52). PB screening on *Plasmodium falciparum* reveals one Digestive Vacuole-Disrupting Molecules for subsequent hit-to-lead generation (53). Novel antifungal agents were discovered by Mayer & Kronstad, (54). In addition, PB was also screened for barber's pole worm (55), across multiple Pathogens (56), Non-tuberculous Mycobacteria species (57), and *Toxoplasma gondii* (58), resulting several novel compounds as a starting point for new drug discovery.

1.7. Rationale of the study

Leishmaniasis is a disease is strongly linked to poverty and 90% of the cases are in the poorest areas of Bangladesh, Brazil, Ethiopia, India, Nepal and Sudan (59). Although the annual burden of leishmaniasis is stable for several years increasing numbers of human immunodeficiency virus (HIV) coinfections, climate change, population mobility, poverty, poor malnutrition and parasite drug resistance make resurgence a possibility (30,31).

The range of drugs available for the treatment of VL is limited. It includes pentavalent antimonials, Amphotericin B deoxycholate (AB), lipid formulations of Amphotericin B (L-AB), miltefosine (MF) and paromomycin (PM); all of which have limitations in terms of toxicity, variable efficacy, price and inconvenient treatment schedules. All are parenteral (AB and its lipid formulations by venous infusion, SbV and PM by intramuscular injection) except for MF which is administered orally (14,22,59).

Due to resistance to drugs, the most widely used of these drugs 'SbV' is now of little use in different parts of endemic region. Resistance to SbV has resulted from several concurrent factors, high drug costs and the absence of functioning health systems have led to poor compliance and incomplete treatments (59). The overall phenomenon of antimonial resistance is multifactorial, increased intracellular thiol levels, reduced uptake of the drug, sequestration and rapid drug efflux are some of the mechanism known for antimony resistance (60).

Resistance to Pentamidine is believed to be caused by reduced uptake and increased efflux (59–61)**.** *L. donovani* miltefosine transporter (LdMT) and the protein LdRos3 has shown to play an important role in resistance to miltefosine (59). Paromomycin, being an aminoglycoside, is at an increased risk of developing resistance; however, clinical resistance to the drug has not been reported as yet (8,59). SbV is still useful in the African endemic region but it is potentially toxic and requires 30 days of painful intramuscular injections. In areas of resistance to SbV, AB is highly effective but requires hospitalization for clinical and laboratory monitoring during one month of treatment (61).

The formulation of L-AB has been a major advance in the treatment of VL since L-AB is very well tolerated and extremely effective, making high-dose short course treatments possible.

However, despite a significant 90% reduction in price, this treatment remains very expensive for endemic countries (62). Miltefosine is highly effective and has the great advantage of being an oral drug but it has to be administered for 28 days and it is also potentially teratogenic and should not be used in women who are pregnant (63,64). PM is effective, safe and very cheap but requires painful intramuscular injections for 21 days when used in monotherapy (65).

The aforementioned side effects and the development of resistance motivate researchers to find alternative safe, effective and affordable medicines and drug combinations for the treatment of leishmaniasis. New candidate drugs can be oral, topical or parenteral, which can cure the lesions quickly without leaving deep scar, shorten treatment cycles and easy to be deployed within primary healthcare systems for self-treatment.

Searching for novel anti-leishmanial leads by phenotypic screening is an important approach to discover and develop drugs against leishmaniasis. PB contains many compounds active against malaria, kinetoplastids and other protozoan parasites. Screening these compounds for leishmaniasis is vital to maximize and exploit the richness of the PB so as to maintain momentum towards discovery of new drugs. Therefore, this study is aimed to Explore effective leads that can comprise a potential combination therapy with improvised pharmacologic property that are essential to fill the translational innovation gaps in leishmaniasis drug discovery.

The findings will be an input to structure-activity relationship studies that can improve certain features of the leads to find candidate molecule for the next step undertakings in drug development. This phenotypic screening project will also allow researchers to understand active compound with a satisfactory cell uptake and open opportunity for further study on new molecular target within Leishmania species.

2. Objective

2.1. General objective

The general objective of this study was to screen Pathogen Box compounds for antileishmanial activity using two stages of leishmania parasites, and to evaluate those leads for their synergistic activity using isobolar analysis.

2.2. Specific objectives

The specific objectives of this study were:

1. To screen the Pathogen Box compounds against promastigotes of *L. aethiopica* and *L. donovani* species.
2. To evaluate the active anti-promastgotes compounds for their activity against intracellular amastigotes of *L. aethiopica* and *L. donovani* species.
3. To evaluate cytotoxicity of compounds against mammalian cells: human THP-1 cell lines, and sheep red blood cells.
4. To determine the selectivity index of the compounds
5. To test for synergism of active compounds

3. Materials and methods

3.1. Materials

3.1.1. Chemical and reagents

Reagents and chemicals used in this work were; methanol (Loba-chemie, India), formaldehyde (Merck, Darmstadt, Germany), resazurin sodium salt (sigma-aldrich, Germany), dimethyl sulfoxide (laborchemikaien GmbH, Germany), triton X- 114 (Sigma-Aldrich laborchemikaien GmbH, Germany). Chloroform (Reagent, Cheshire, UK), absolute ethanol (zfine chemicals, Ethiopia), distilled water (LRDL, Leishmania laboratory), Giemsa powder (Fisher scientific, UK), tryptan blue stain (Grand island, USA), potato starch powder (Sigma-Aldrich Laborchemikalien GmbH, Germany), phosphate buffer saline (Gibco, USA).

3.1.2. Culture medium

Roswell Park Memorial Institute (RPMI-1640) (sigma-Aldrich, UK), Medium-199 (M199) (Sigma-Aldrich, US), Minimum essential medium (MEM) (Sigma-Aldrich, USA), and Heat Inactivated New Born Calf Serum (HINBCS) (Sigma-Aldrich, USA) penicillin-streptomycin solution (Sigma-Aldrich, USA) and HEPES (Sigma-Aldrich, USA), were used to make complete culture medium. D -glucose, calcium chloride dihydrate, potassium chloride, sodium bicarbonate, potassium dihydrogen phosphate, sodium chloride (all from Sigma-Aldrich, USA), Sheep and Rabbit blood, were used to prepare NNN media and lock's solution (Annex 3).

3.1.3. Reference drugs and test compounds

Amphotericin B, Pentamidine isothionate, Miltefosine (all from Sigma-Aldrich Che. Co., St. Louis, USA) were used as a reference drugs, and are used to check sensitivity of parasites (promastigotes and amastigotes). The PB was supplied in sealed plates containing frozen 10 mM dimethyl sulfoxide (DMSO) solutions (10μL of each compound) in 96-well plates, along with handling instructions. The compound number, structure, canonical SMILES, biological data, and physicochemical parameters are also released at (http://www.pathogenbox.org/about-pathogen-box/) in excel files.

3.1.4. Test strains, cell line and laboratory animals

Leishmania isolates, *L. aethiopica* (579/17) *and L. donovani* (AM1295) were obtained from Leishmaniasis Research and Diagnostic Laboratory (LRDL), at the department of Microbiology,

Immunology and Parasitology, Addis Ababa University. *L. aethiopica* strain (579/17) was clinically isolated from a 50 year old female patient who resides in Ambo, Western Shoa zone, Oromia region, Ethiopia. The VL strain AM1295 was isolated from a 33 year old female patient residing in Liben district, Oromia region, Ethiopia. THP-1 cell lines were kindly provided by Dr. Adane Mihret, Armaueur Hansen research institute (AHRI). White Swiss albino mice were obtained from Addis Ababa University animal house.

3.2. Methods

3.2.1. Culture Conditions

3.2.1.1. Leishmania parasite isolation and culture

The parasites used in this study were selected among many other leishmnaia promastigote cultures. Isolated parasites were cultured in NNN media. Suitable strains were selected based on their response to reference drugs (purchased reference drugs) and their adaptability to liquid media. Accordingly, two strains one from VL and another from CL were selected and cultured separately. Cultures were carefully monitored and timely subcultured using standard laboratory protocols.

For the purpose of the assay, logarithmic stage parasites were inoculated in to M199 liquid media. The culture was carefully monitored every day to ensure healthy growth. Later, promastigotes at their logarithmic stage were taken from liquid media for every anti-promastigotes biological assay, while stationary phase promastigotes were used for macrophage infection.

3.2.1.2. THP-1 Cell line cultures

THP-1 cells were cultured in RPMI 1640 Medium (Sigma Aldrich, UK) supplemented with 10% Heat Inactivated New Born Calf Serum (HINBCS), 100 U penicillin and 100 µg streptomycin/ml in humidified, 5% CO_2 and incubator at 37°C (66–68). These cell lines were used to determine cytotoxic effect of selected compounds.

3.2.1.3. Intra-peritoneal macrophage collection and culture

Macrophage collection from White Swiss albino mice was done according to Maes et al. (69), Zhang et al. (70) and Vermeersch et al. (71). Briefly, Young Mice (6-8 weeks old) were isolated

and maintained separately. The skin disinfected by 70% ethanol and mice were injected with 2% freshly prepared starch in to the peritoneal cavity using 25 G needle. Injected mice kept under appropriate condition. After two days, mice were sacrificed with ether and the skin underlying the peritoneal cavity was sheared aseptically to expose intact peritoneum. Then 10 ml of sterile ice cold phosphate-buffered saline (PBS) with 3% HINBCS was injected into the peritoneal cavity. Peritoneal wall was massaged carefully to dislodge attached macrophages from peritoneal wall. Finally, macrophages were harvested by drawing 6-8 ml exudates of the PBS. The contents were transferred into sterile 15 ml test tube, and centrifuged at 450g for 10 minutes. The resulting pellet was re-suspended in complete Minimum Essential Medium (MEM) containing 10% HINBCS, 25mM HEPES, 2mM L-glutamine and 100 U penicillin and 100 µg streptomycin/ml. The host cells counted and adjusted accordingly using haemocytometer. Then 3x 10^5 macrophages were inoculated in every well of either 24 well micro culture plate or 16 well lab-tek chamber slides.

3.2.2. Preparation of test and reference compounds

The master plates supplied by MMV were prepared based on the given recommendation. The 10 mM stocks were diluted to a concentration of 1 mM (the initial 10 µL of stock solution into 90 µL of DMSO). The first dilution was used to generate 10 plates containing 10 µL solutions at 1 mM concentration which is done to avoid unwanted multiple freeze/thawing cycle during the experiment. The stocks together with the supplied master plate were stored at -20 ^{0}C till used. Test compounds were prepared in DMSO and intermediate dilutions were made by complete media. Purchased reference drugs were prepared following the guidelines of the manufacturers. In all assays except for hemolytic and THP-1 cytotoxicity assays concentration of DMSO was kept at 1% and below to prevent its negative outcome in cell viability.

3.3. Biological Assay

3.3.1. General work flow

The compounds were first tested *in vitro* on the two Leishmania species. The most active compounds were then selected. An activity of >70% inhibition on promastigotes used as a cut-off point. IC_{50} values determined for selected compounds. Cytotoxicity studies and data mining were also done to evaluate safety and physico-chemical property of the compounds.

Finally, selected compounds were evaluated on peritoneal macrophages harboring Leishmania amastigotes (Figure 6).

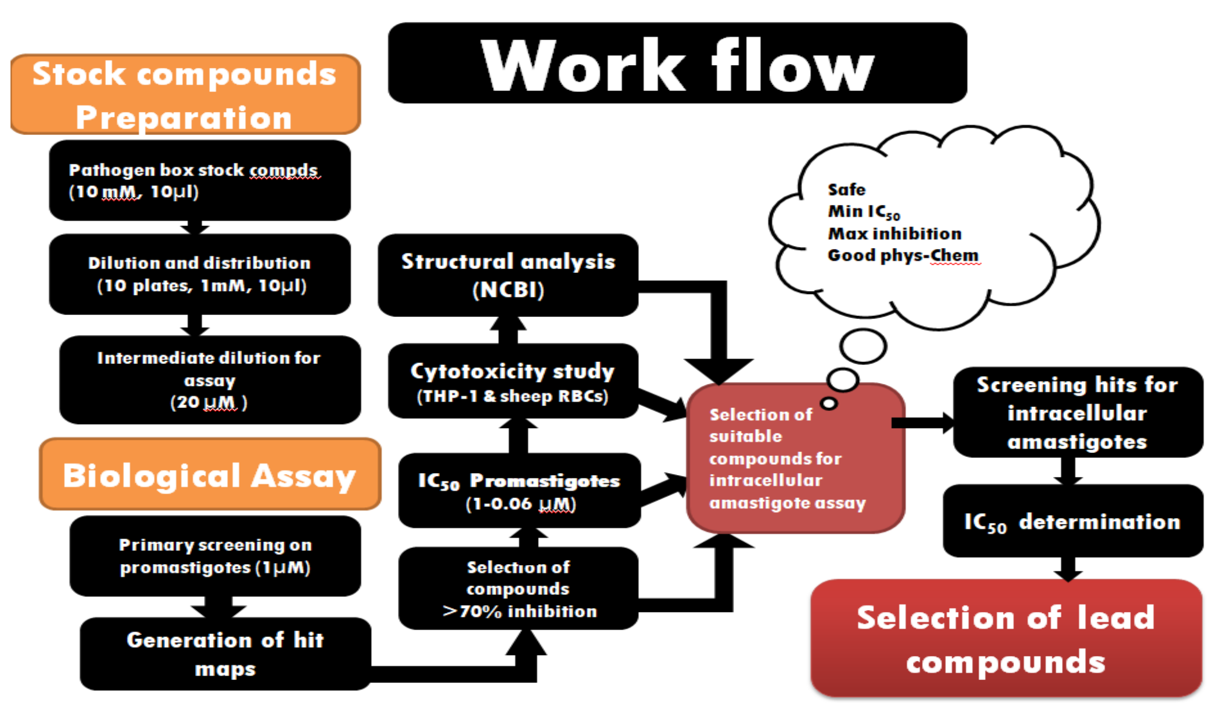

Figure 6: Detailed workflow and hit to lead selection procedures used in the Pathogen Box against *Leishmania aethiopica* and *Leishmania donovani* isolates.

3.3.2. Anti-promastigotes assay

3.3.2.1. Primary screening (determination of percent inhibition)

Primary screening of the library, consisting of 400 compounds against *L. donovani* and *L. aethiopica* was done using standard screening methodologies. For all biological assays stock compounds passed through three successive dilutions to reach to 1 µM final concentration. Test compounds were diluted in 96-well microculture plate containing 100 µl of complete Medium 199 (M199) supplemented with 15% HINBCS, 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin solution (Sigma-Aldrich, USA) (68,72–74). Test compounds were added in triplicate to achieve a concentration of 2 µM in 100 µl of complete media. Then 100 µl of suspension of parasites (1 x 10^6 promastigotes/ml of *L. aethiopica* and *L. donovani*) obtained from a logarithmic growth phase of previous culture was added to each well and contents of the plates maintained at 22°C (*L. aethiopica*) and 26 °C (*L. donovani*) for 72 hrs. The activity of the compounds was observed using an inverted microscope (72).

The activity of compounds to inhibit the parasite growth was determined using Alamar Blue assay. The oxidation reduction indicator Alamar Blue was used for colorimetric determination of mammalian and yeast cell viability and proliferation. In the living cell, Alamar Blue is reduced thereby changing its color from blue to red (75,76). The assay involves the addition of 10% of fluorochrome resazurin solution (12.5 mg dissolved in 100 ml of PBS) after 68 h of incubation. The fluorescence intensity after a total incubation time of 72h was estimated by Multi-label plate reader at excitation wavelength of 530 nm and emission wavelength of 590 nm (72,75,77). Following this primary screening test compounds which have more than 70% inhibition were selected for IC_{50} determination.

Blank wells containing complete M199 medium with no parasites or test compounds used as negative control (100% inhibition) and other wells containing freely growing promastigotes in complete medium with no test compounds were used as positive control (0% inhibition) (78–80).

3.3.2.2. Determination of 50% inhibitory concentration against promastigotes

Selected compounds from the primary screening were collected and pooled in to one plate. Pooled compounds (10µl, 1 mM solution) were marked and diluted by 90 µl DMSO to make 100 µL of 100 µM solution. After this point, dilutions were made by complete media to prevent entry of high level of DMSO in to the cell culture. An intermediate dilution was made by diluting 20 µl test compound in to 80 µl fresh media to get 20 µM temporary stock. From this stock, 20 µL test substance was pipetted into 180 µL complete medium to produce 2 µM in 200 µL media. This concentration was the highest initial concentration. From this well 100 µL was taken in to subsequent wells composing 100 µL of complete medium (two fold serial dilution). A total of 5 dilutions were made (1 - 0.0625 µM) to establish a full dose titration and determination of the IC_{50}. All dilutions were done carefully by avoiding bubble formations. Parasite cultures containing 1x 10^6/ml logarithmic phase promastigotes were finally added to each well. Table 1 show the protocol used to prepare a serial dilution of test substance in 200 µl volume media containing parasites as the solvent.

Table 1: Serial dilution of stock compounds for IC_{50} determination

Dilution step	Pathogen Box test substance (µL)	Complete medium (µL)	Compounds in 100µL (µM)
1	20 (from 20 µM stock)	180 (1st well)	2
2	100 (from dilution 1)	100 (2nd well)	1
3	100 (from dilution 2)	100 (3rd well)	0.5
4	100 (from dilution 3)	100 (4th well)	0.25
5	100 (from dilution 4)	100 (5th well)	0.125

IC_{50} values were computed using Graph Pad Prism software. The controls were blanks containing complete M199 medium with no parasites (100 % inhibition) and logarithmic phase parasite without test compounds (0% inhibition).

3.3.3. Cytotoxicity assay

Selected Pathogen Box compounds from the previous assay were evaluated *in vitro* for their toxicity to human cells using the human monocytic THP-1 cell line. Their hemolytic activity was also assessed using sheep red blood cells (RBCs). From these data the concentrations which killed 50% of THP-1 cells (CC_{50}) was calculated.

3.3.3.1. Haemolysis test

Hemolysis test was done according to a method described by Mahmoud et al. (66) and Esteves et al. (67). Briefly, two ml freshly defibrinated sheep blood was added to 48 ml PBS (pH 7.2) and Centrifuged at 1000g for 10 min. The solution was then washed twice and the supernatant was removed resulting formation of approximately 1 ml RBC pellets. The resulting pellet was then re-suspended in 49 ml PBS (pH 7.2) to make 2% blood suspension. Then, 200 µl of the blood suspension was added to eppendorf tube containing test substances in serial dilution (25- 0.04 µM). The blood suspension and the solution were carefully mixed and incubated at 37 ^{0}C for two hours. The mixture was then centrifuged at 1000g for 10 minutes and 75 µL from the resulting supernatants of each tube was collected and placed in 96 well plates and absorbance of the supernatant was measured at 540 nm. Tests were performed in triplicate. Triton X-114 was used as a positive control (5µl/ml). It was prepared by adding 50 µl blood to 100 µl Triton X-114 and incubated at 37 ^{0}C for 30 min. RBC suspension with 2.5 % DMSO was used as a negative control.

3.3.3.2. THP-1 cells viability assay

Evaluation of cytotoxic effects of selected compounds in THP-1 cell lines was done according to Habtemariam et al. (51) and Esteves et al. (68). Briefly, in 96-well plates containing 50 µl serially diluted test molecules, pre-cultured 50 µl suspensions of 1 x 10^6 THP-1 cells were added. Contents of the plates were then incubated for 68 hrs at 37°C in a 5% CO_2 air mixture. After 68 h of incubation, 10µl of resazurin solution (12.5 µg dissolved in 100 ml of PBS, pH=7.2) was added into each well and the fluorescence intensity was measured after a total incubation time of 72h using multilabel plate reader at excitation wavelength of 530 nm and emission wavelength of 590 nm. Several readings were taken between 72 h and the next 24 h thereafter. All results were taken after 24 h of resazurin addition. Cell viability (%) was calculated for each concentration using the following formula:

$$\text{Cell viability } (\%) = \frac{(\text{absorbance in duplicate drug wells} - \text{ average blank wells})}{(\text{Average absorbance control wells} - \text{average blank wells})} * 100$$

The 50% lethal concentration which kills 50% of the cells (CC_{50}) values for compounds was calculated from sigmoidal dose response curves using computer software Graphpad prism 7.0. The controls were blanks containing complete RPMI medium without THP-1 cells (100 % inhibition) and logarithmic THP 1 cells without test compounds (0% inhibition).

3.3.4. Selection of suitable compounds for intracellular amastigote assay

To select potential Hits for further intracellular amastigote assay, the Japanese Global Health Innovative Technology (GHIT) disease specific criteria for hits and leads targeting Leishmania parasites was used. Compounds were considered to be safe if they have greater than 100- fold selectivity (SI>100) in killing pathogens as opposed to mammalian cells (83).

Physico-chemical data were collected from National Centre for Biotechnology Information (NCBI). Collected data were used to measure fitness of test compounds for cell membrane penetration. To evaluate suitability of these compounds for intracellular amastigote assay Lipinski's rule of permeation and absorption was used as a guideline. In additional to the above criteria the IC_{50} value of selected compounds and percentage of inhibition against promastigotes assay were considered for selection.

3.3.5. Intracellular amastigote assay

Macrophage collection was made as mentioned in section (3.2.1.3). Collected Macrophages were counted using haemocytometer and their total number was adjusted to 1x 10^6 cells per ml in complete MEM. Approximately 3x 10^5 macrophages were seeded in 24-well plates containing removable microscopic slides. Cells were allowed for adherence for at least 12 hours at 37°C in 5% CO_2. Non-adherent cells were washed twice with pre-warmed complete media and incubated overnight in fresh media. Following overnight incubation, adherent cells were infected with late stationary stage *L. donovani* and *L. aethiopica* promastigotes with a parasite-to-cell ratio of 10:1 and incubated further for 12 hrs. After removal of non-internalized promastigotes by extensive washing with MEM, cultures were allowed to rest for about 4 hrs (66,84,85) and incubated with or without test drugs for three days at 31 ^{0}C (*L. aethiopica*) or 37^{0}C (*L. donovani*), 5% CO_2 and 95% relative humidity. Amphotericin B and Pentamidine were used as reference drugs to check sensitivity of the parasites.

Following a 72 h of incubation, the media was removed and slides were washed with PBS (pre-warmed at 37°C), fixed with methanol and stained with Giemsa (10%) for 15 minutes. The number of amastigotes was determined by counting amastigotes in at least 50 macrophages in duplicate cultures. Evaluation of anti-amastigotes activity was done by assessing the infection rate and parasitic load in both treated and untreated groups. The total parasite burdens was microscopically assessed and compared to the burdens in the untreated infected controls (86). Infection was judged to be adequate if more than 70% of the macrophages present in the untreated controls were infected. The total actual parasite burdens were calculated using the infection index we refer to as the associate index. The infection index for each well in duplicate was determined by multiplying the percentage of infected macrophages (IR) with the average number amastigotes in infected cell. The index can show the number of parasites that actually infected the macrophages (87–89).

$$Infection\ index = \frac{\text{Number of infected macrophage}}{total\ macrophage\ counted}\ x\ \frac{\text{number of amstigotes in 50 infected macrophages}}{total\ infected\ macrophages\ counted}$$

In other words, the first half of the equation gives the IR and the second part gives average parasite load found in infected macrophages. Final results for analysis were expressed as percent of reduction of the parasite burden of treated wells in comparison with those obtained with the

controls. The IC_{50} of each test compound was defined as the inhibitory concentration of test compounds that reduces amastigotes density (number of amastigotes per infected macrophages by 50% (84,85,87,90).

3.3.6. Selectivity Index/ SI

The SI was determined using 50% cytotoxic concentrations of the normal cell line (CC_{50} of THP-1) and the IC_{50} of parasites (both promastigote and amastigotes). The selectivity of the compound in killing pathogens as opposed to mammalian cells was assessed by the following formula (58,77,91):

$$\text{selectivity index (SI)} = \frac{\text{CC50 THP1 cells}}{\text{IC50 protozoa}}$$

3.3.7. Evaluation of synergistic activity of molecules

The presence or absence of *in vitro* drug interactions was assessed using a modified fixed-ratio isobologram method as described by Chou et al. (78) and others (92–97). Individual drugs IC_{50}s were used to determine the maximum concentrations of individual drugs combined together. This was done to ensure that the IC_{50} fell near the midpoint of a five point serial dilution.

Since synergism/antagonism quantification is based on the principle of mass-action law and determined by the CI values, the dose-response effect of each drug alone was important. Selected compounds with common reference drugs were mixed as illustrated in Table 2.

Table 2: Layout of a combination experiment for synergistic effect of two drugs

Drug 1	4(**IC_{50}**)= A	2(**IC_{50}**) =C	**(IC_{50})= E**	0.5(**IC_{50}**) =G	0.25(**IC_{50}**) =I
Drug 2	4(**IC_{50}**) = B	2(**IC_{50}**) =D	**(IC_{50})= F**	0.5(**IC_{50}**) =H	0.25(**IC_{50}**) =J
Combination	**A+B**	**C+D**	**E+F**	**G+H**	**I+J**

The CI value was then expressed as CI<1, =1, and >1 which indicate synergism, additive effect, and antagonism, respectively.

3.3.8. Equipment

The following equipment were used: 25cm^2 polystyrene sterile tissue culture flasks (Corning incorporated, USA), 16 well chamber slides, 24-well and 96- well cell culture plates (Corning, USA), micro pipettes (Pipetman ultra), multichannel pipettes (Hamilton), haemocytometer

(Improved Double Neubauer type), eppendrof tube (generic), compound microscope and inverted microscope (Olympus and Labomed), thermoshake (Gerhardt), autoclave (Timo), oven drier (precision), biosafety cabinet with UV (Laboculture ESCO class IIA), analytical balance (Mettler Toledo), microculture plate reader (victor 3 Perkin Elmer), Flouroscan Ascent (Thermo electron), carbon dioxide (CO2) Incubator (Thermo Electron), centrifuge (eppendorf 5804R), digital water bath (Julabo TW20), vortex (whirl VIB2), pipette tips (eppendrof), pipette tips rack (generic), microslides (Sunbeam), syringes (27, 25 and 20 guages), table-top vacuum filter (0.22µm) (micropore, Brazil), aluminium foil, parafilm, glass vials, crucible dishes, clamps.

3.4. Data management and statistical analysis

All data were taken in a prepared log sheet and entered in Microsoft Excel spread sheet. Raw data were normalized against that of the positive and negative controls to remove plate-to-plate variation. The Percent of inhibition for test and reference compound was expressed as mean values ± standard deviations. The 50% median inhibitory concentrations were expressed as IC_{50}± confidence interval (95%CI). IC_{50} value was determined by non-linear regression analysis using GraphPad Prism latest version 7.00 (GraphPad software, inc. 2016). Drug combination effect of selected compounds with the reference drugs was made with isobologram method and analysis was made using compusyn (CompuSyn 1.0, ComboSyn, Inc., 2005).

4. Results

4.1. Primary screening results (Percent inhibition)

The primary screening was done for all compounds in the Pathogen Box, regardless of their disease set. All compounds were screened for activity against promastigote stage of *L. aethiopica and L. donovani at* 1µM. Hit maps were generated for the five 96-wells pathogen box plates. Compounds classified under different ranges of % inhibition, as presented in Figure 6 & 7.

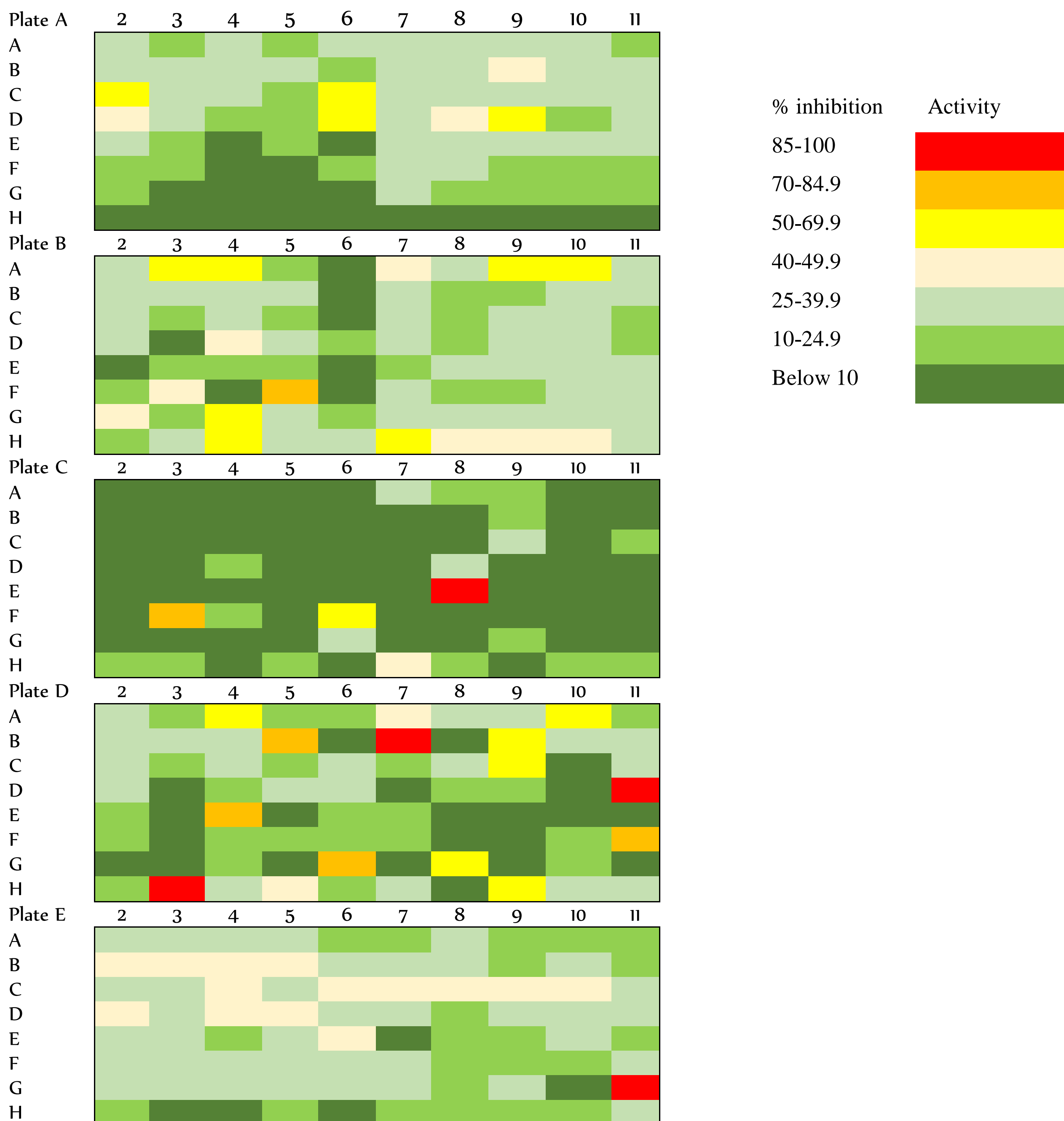

Figure 7: Hits map for anti-promastigote activity on *Leishmania aethiopica*

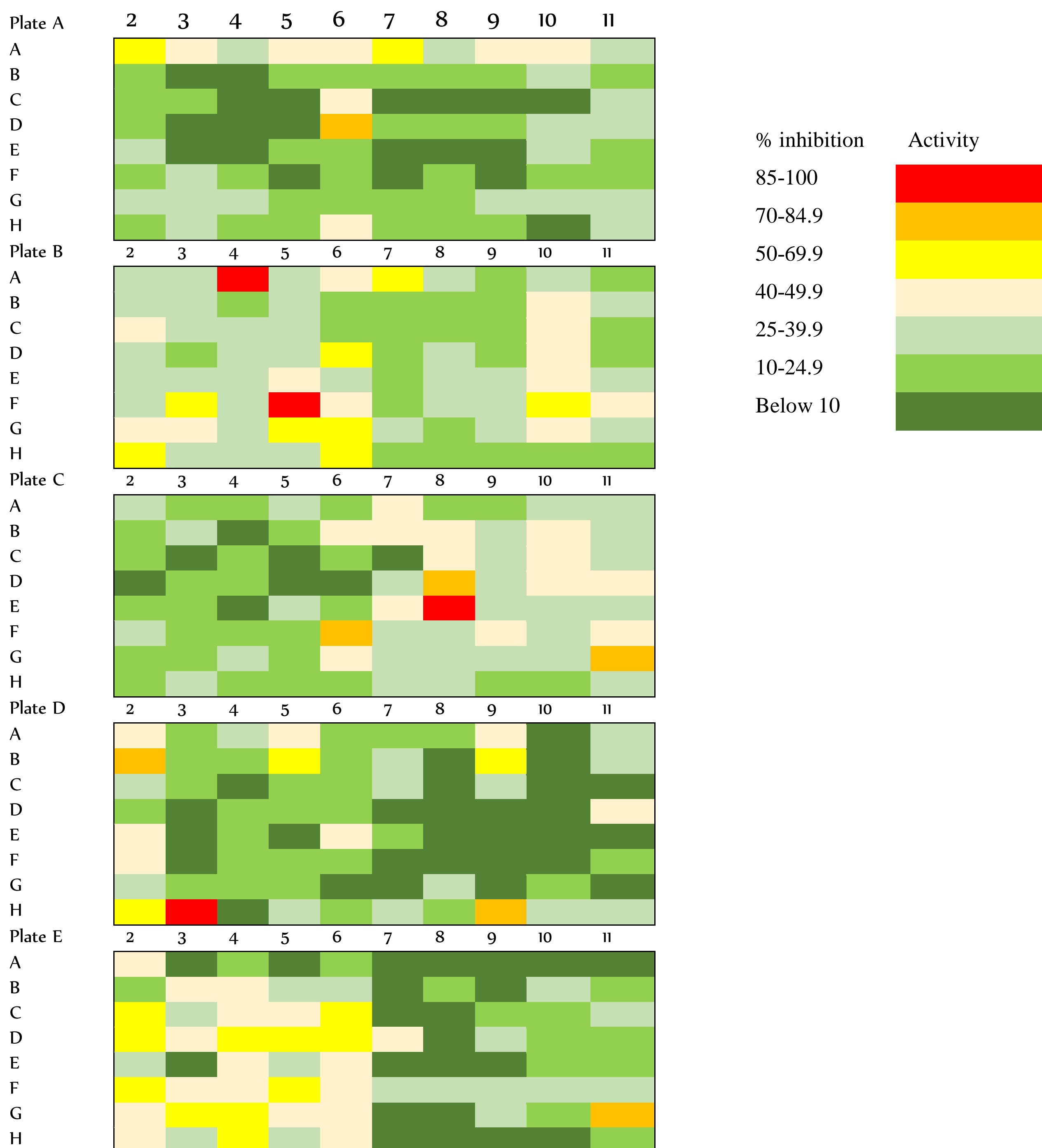

Figure 8: Hit map for anti-promastigote activity on *Leishmania donovani*

The map was used to select active compounds which demonstrated above 70% inhibition (Red and Orange colour) for estimation of median inhibitory concentration (IC_{50}). The Pathogen Box compounds shown in the above figures 6 and 7 were classified using different range of percentage of inhibition. Tables 3 and 4 contain list of selected compounds for IC_{50} determination.

Table 3: Selected molecules for further investigation on *Leishmania aethiopica* promastigotes

Plate & location	MMV ID	Molecular Weight	Molecular Formula	Molecular structure
B-F5	MMV637229	459.97	$C_{21}H_{26}NOCl$	
C-E8	MMV690102	417.46	$C_{22}H_{23}N_7O_2$	
C-F3	MMV688179	476.18	$C_{18}H_{16}N_6OCl_2$	
D-B5	MMV024829	380.54	$C_{23}H_{32}N_4O$	
D-B7	MMV022029	449.62	$C_{26}H_{31}N_3O_2S$	
D-D11	MMV024406	428.32	$C_{21}H_{19}N_5OCl_2$	
D-E4	MMV085071	348.41	$C_{19}H_{20}N_6O$	
D-F11	MMV687812	534.51	$C_{24}H_{26}N_8O_2F_4$	
D-H3	MMV022478	545.93	$C_{23}H_{21}N_6OCl$	
D-G6	MMV663250	366.90	$C_{21}H_{23}N_4Cl$	
E-G11	MMV688415	433.54	$C_{26}H_{31}N_3O_3$	

Table 4: Selected molecules for further investigation on *Leishmania donovani* promastigotes

Plate & location	MMV ID	Molecular weight	Molecular formula	Molecular structure
B-F5	MMV637229	459.97	$C_{21}H_{26}NOCl$	
C-D8	MMV690103	349.43	$C_{19}H_{23}N_7$	
C-E8	MMV690102	417.46	$C_{22}H_{23}N_7O_2$	
C-F6	MMV688262	534.48	$C_{25}H_{25}N_4O_6F_3$	
C-G11	MMV688703	335.42	$C_{21}H_{22}N_3F$	
D-B2	MMV011511	367.54	$C_{23}H_{33}N_3O$	
D-H3	MMV022478	545.93	$C_{23}H_{21}N_6OCl$	
D-H9	MMV675968	359.81	$C_{17}H_{18}N_5O_2Cl$	
E-G11	MMV688415	433.54	$C_{26}H_{31}N_3O_3$	

Sixteen compounds (9 compounds for VL, 11 for CL) were selected; out of which 4 compounds had >70% inhibitory effect on both strains. These compounds were primarily designed and tested for different disease sets. Seven of them were from malaria disease set (MMV022478,

MMV011511, MMV024829, MMV022029, MMV024406, MMV085071 and MMV663250), Four compounds were from kinetoplastids disease set (MMV690102, MMV688415, MMV688179 and MMV690103), two compounds were from tuberculosis disease set (MMV687812, MMV688262), and the rest MMV675968, MMV688703 and MMV637229 were obtained from *Cryptosporidiosis, Toxoplasmosis* and *Trichuriasis* disease sets respectively.

4.1.1. Median inhibitory concentration (IC_{50})

4.1.1.1. Median inhibitory concentration (IC_{50}) against promastigotes of L. aethiopica

The IC_{50} of selected compounds was determined against promastigote stage of *L. aethiopica* and *L. donovani* isolates in two separate experiments (Table 5 and Table 6). Among the tested compounds MMV024406 and MMV688415 demonstrated IC_{50} of 0.010 (95% CI=0.081 - 0.012) and 0.162 (95% CI= 0.140-0.187) µM, respectively.

Table 5: Activity of selected compound against of *Leishmania aethiopica* promastigotes

Plate & well location	MMV ID	Experiment 1 IC50 (95% CI), µM	R^2	Experiment 2 IC50 (95% CI) µM	R^2
B-F5	MMV637229	0.552 (0.427 -0.748)	0.814	0.398 (0.341- 0.467)	0.936
C-E8	MMV690102	0.214 (0.168 -0.268)	0.891	0.277 (0.240- 0.318)	0.956
C-F3	MMV688179	0.316 (0.185 - 0.522)	0.901	0.378 (0.321- 0.447)	0.895
D-B5	MMV024829	0.207 (0.177 - 0.240)	0.942	0.506 (0.352- 0.683)	0.771
D-B7	MMV022029	0.294 (0.244- 0.357)	0.912	0.319 (0.281- 0.365)	0.923
D-D11	MMV024406	0.010 (0.081 - 0.012)	0.986	0.018 (0.014- 0.022)	0.972
D-E4	MMV085071	0.206 (0.117 - 0.325)	0.639	0.346 (0.265- 0.472)	0.830
D-F11	MMV687812	0.373 (0.283 - 0.502)	0.826	0.398 (0.341- 0.467)	0.936
D-G6	MMV663250	0.473 (0.396 - 0.580)	0.929	0.678 (0.441- 1.380)	0.637
D-H3	MMV022478	0.326 (0.195 - 0.532)	0.641	0.309 (0.271- 0.355)	0.955
E-G11	MMV688415	0.162 (0.140 -0.187)	0.953	0.130 (0.104 -0.160)	0.894
Amp B	0. 106 (0.66- 0.140)				
Miltefosine	3.01 (1.63- 5.76)				
Pentamidine	1.31 (0.728- 1.92)				

95% CI= 95% confidence interval, IC_{50}= Median inhibitory concentration, R^2= Regression coefficient

4.1.1.2. Median inhibitory concentration (IC_{50}) against L. donovani promastigotes

Correspondingly, IC_{50} of selected compounds was determined using the same fashion in *L. donovani* promastigotes. Four compounds (MMV690102, MMV688262, MMV688415 and MMV022478) were selected for further study in intracellular of *L. donovani* amastigotes following their inhibitory activity against the promastigote stage.

Table 6: Activity of selected compound against *Leishmania donovani* promastigotes

Plate & well location	MMV ID	Experiment 1 IC_{50} (95%CI), μM	R^2	Experiment 2 IC_{50} (95%CI), μM	R^2
B-F5	MMV637229	0.371 (0.266- 0.551)	0.7689	0.141 (0.110- 0.175)	0.904
C-D8	MMV690103	0.491 (0.353- 0.767)	0.7567	0.168 (0.109- 0.241)	0.739
C-E8	MMV690102	0.012 (0.002- 0.022)	0.7435	0.055 (0.030- 0.078)	0.794
C-F6	MMV688262	0.236 (0.192- 0.291)	0.9001	0.262 (0.204- 0.338)	0.8895
C-G11	MMV688703	0.175 (0.035- 0.221)	0.873	0.361 (0.289- 0.458)	0.8665
D-B2	MMV011511	0.188 (0.127- 0.261)	0.769	0.337 (0.247- 0.474)	0.7913
D-H3	MMV022478	0.295 (0.213- 0.401)	0.7944	0.124 (0.042- 0.213)	0.5551
D-H9	MMV675968	0.295 (0.217- 0.412)	0.8174	0.299 (0.219- 0.420)	0.814
E-G11	MMV688415	0.224 (0.129- 0.369)	0.6237	0.162 (0.117- 0.214)	0.8697
Amp B	0.135 (0.102- 0.169)				
Miltefosine	4.23 (1.82- 8.95)				
Pentamidine	0.651 (0.314- 1.21)				

95% CI= 95% confidence interval, IC_{50}= Median inhibitory concentration, R^2= Regression coefficient

4.1.2. Cytotoxicity assay

In this study, safety of selected compounds on mammalian cell was assessed on Sheep blood RBCs and human monocytes (THP-1) cells in two-fold serial dilution (25- 0.3 μM). All, except two compounds have less than 5% hemolysis at 25 μM and have limited hemolytic activity at therapeutic concentrations. These two compounds (MMV675968 and MMV6884150) produced 6.9% and 5.3% haemolysis respectively.

The tested Pathogen Box compounds did not show considerable cytotoxic activity on THP-1 cell line at the highest tested concentration (25 μM). One compound (MMV690102) from the

Kinetoplastid disease set demonstrated some degree of cytotoxicity (CC_{20}=25 µM). Extrapolated result indicates CC_{50} value of 36.55 µM (95%CI: 29.86-45.36). On the other hand a biological data provided by MMV showed that MMV690102 inhibited half of MRC-5 and PMM cells at 5.4 µM and 4 µM µM, respectively. From similar disease set (kinetoplastids) all compounds with the exception of MMV690103 are proved to be safe on MRC5 and PMM. MMV690103 demonstrate inhibitory activity on MRC5 (CC_{50}=7.1 µM) and PMM (CC_{20}=8.0 µM) (Annex 1B).

All compounds from the malaria disease set except MMV022478 didn't show considerable cytotoxicity to HepG2 cells. MMV022478 had a CC_{20} of 0.764 µM, which is very close to the IC_{50} required to inhibit P. *falciparum* gametocytes (0.6 µM) (Annex 1A). Two compounds from two different disease sets are reported to affect HepG2 cells. MMV675968 (cryptosporidiosis disease set) showed CC_{20} at 3.6 µM and MMV687812 (tuberculosis disease set) affects half of the cells at 3.9 µM (Annex 1E). The remaining compounds did not have considerable cytotoxic reports on mammalian cells at concentration they were tested (Annex 1).

4.1.3. Selectivity index

SI index was computed for one compound because the CC_{50} value of the remaining compounds could not be determined. The calculated SI of MMV690102 with respect to THP-1 cells was more than 700 and 132 for *L. donovani* and *L. aethiopica* strains, respectively. The Cytotoxicity effect of this compound on other mammalian cells was provided by MMV. By taking this data, SI of MMV690102 on MRC-5 and PMM cells was calculated to be 300 and 222, respectively. The SI index was computed by taking the IC_{50} (0.018 µM) from the present study.

4.2. Selection of suitable 'Hits' for intracellular amastigote assay

After determining the IC_{50} of selected compounds on promastigotes and their cytotoxivity activity on mammalian cells, selected compounds examined for their physico-chemical characteristics. The examination was done to validate selected compounds for intracellular amastigotes assay. Relevant physico-chemical data were gathered from National Centre for Biotechnology Information (NCBI) and a reference table was constructed as shown in Table 7.

Table 7: Relevant physico-chemical information of selected compounds

S.N.	MMV ID	R1	R2	R3	R4	R5	R6	Reference
1	MMV011511	367.5	1	4	8	3.9	27.7	(98)
2	MMV022029	449.6	2	5	8	4	69.8	(99)
3	MMV022478	432.9	2	5	4	3.2	74.6	(100)
4	MMV024406	428.3	1	5	4	3.9	61.4	(101)
5	MMV024829	380.5	1	4	6	4.4	48.5	(102)
6	MMV085071	348.4	0	7	4	1.3	67.3	(103)
7	MMV637229	343.9	0	2	6	5	12.5	(104)
8	MMV663250	366.1	1	3	5	-	33.1	(105)
9	MMV675968	359.1	3	7	5	2.9	108	(106)
10	MMV687812	534.5	2	13	9	2.9	122	(107)
11	MMV688262	534.4	0	11	7	5.6	104	(108)
12	MMV688415	433.5	2	6	6	2.3	69.1	(109)
13	MMV688703	335.1	1	3	3	3.6	31.9	(110)
14	MMV688942	337.4	1	4	6	4.2	60.2	(111)
15	MMV690102	417.4	2	9	6	3.3	125	(112)
16	MMV690103	349.4	2	7	3	3.2	107	(113)

Description: R1=Molecular weight (g/mol), R2=H^+ bond donor, R3=H^+ bond receiver, R4=Rotatable bonds, R5=XLogP3-AA, R6=Polar surface area (A^2)

Having established the table, all candidate compounds were weighed against Lipinski's rule of absorption and permeation. In view of that, MMV687812, MMV688262 (both from TB disease set) were slightly above the 500 g/mol molecular weight and the 10 hydrogen bond receiver count.

4.3. Intracellular amastigotes assay

4.3.1. Intracellular amastigotes assay against *L. aethiopica*

The effect of six compounds on intracellular amastigotes was assessed. Experiments involving MMV022029 and MMV024406 were unable to produce IC_{50} at the concentration ranges tested, the highest concentration being (1 μM). Therefore they were replaced by MMV085071 and MMV022478. Of these screened compounds MMV690102 was found to be a very potent inhibitor of *L. aethiopica* amastigotes. Table 7 contains the activity of six compounds on intracellular *L. aethiopica* amastigotes.

Table 8: Activity of selected compound against amastigote stage of *Leishmania aethiopica* Isolates

Plate &well location	MMV ID	IC_{50} (95% CI) [µM]	R^2
D-E4	MMV085071	0.168 (0.127- 0.220)	0.9083
E-G11	MMV688415	0.354 (0.269- 0.481)	0.907
C-E8	MMV690102	0.057 (0.031- 0.082)	0.8786
D-H3	MMV022478	0.431 (0.267- 844.2)	0.7522
D-B7	MMV022029	> 1	
D-D11	MMV024406	>1	
Amp B	0.128 (0.110- 148)		
Pentamidine	1.505 (1.135- 1.978)		

Description: 95% CI= 95% confidence interval, IC_{50}= Median inhibitory concentration, R^2= Regression coefficient

4.3.2. Intracellular amastigotes assay against *L. donovani*

Among the top ten compounds identified from the primary screening, four compounds were selected for further intracellular amastigotes inhibition assay on *L. donovani*. Among these compounds, MMV690102 and MMV688262 (delamanid) were found to be potent inhibitors. Delamanid and MMV690102 reduced 50% of amastigotes population at 0.053 µM and 0.06 µM respectively (Table 8). These values are comparable to the IC_{50} of Amphotericin B (0.076 µM).

Table 9: Activity of selected compound against intracellular-amastigotes of *Leishmania donovani* Isolates

Plate & well location	MMV ID	IC_{50} (95% CI) [µM]	R^2
CF6	MMV688262	0.053 (0.044- 0.061)	0.9791
CE8	MMV690102	0.060 (0.047- 0.072)	0.9618
EG11	MMV688415	0.704 (0.530- 0.858)	0.9523
DH3	MMV022478	0.694 (0.560- 0.822)	0.9708
Amp B	0.076 (0.051-0.092)		
Pentamidine	1.83 (0.662-2.99)		

Description: 95% CI= 95% confidence interval, IC_{50}= Median inhibitory concentration, R2= Regression coefficient

4.3.3. Synergistic effect of lead compounds with reference drugs

Based on the performance in the intracellular amastigotes assay, highly active compounds were selected and synergistic effects with commonly used drugs were evaluated. The two combinations were tested in the ratio of 1:2 (MMV690102 and pentamidine) and 1:1

(MMV688262 and Amphotericin B). As it is shown on Figure 7, the combination index value for combination of MMV690102 and pentamidine (CE8PNT) was greater than 2 indicating the two compounds have antagonistic effect.

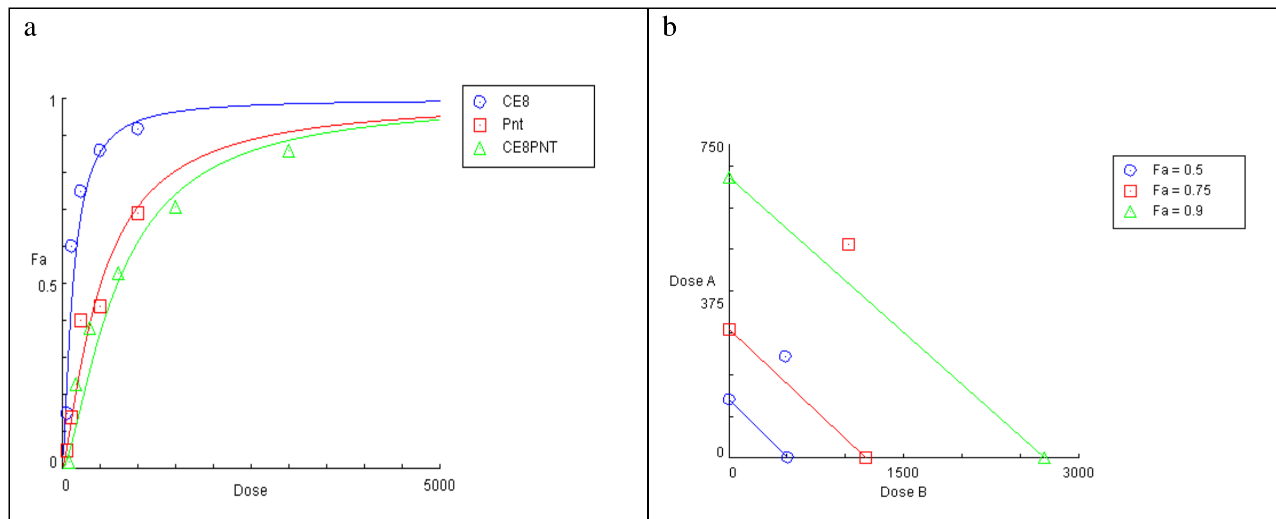

Figure 9: Activity graph: a) Dose-Effect Curve, b) Isobologram for Combination: MMV690102-pentamidine (CE8PNT [1:2])

Similarly, Interaction between MMV688262 [C-F6] and Amphotericin B [AmB] was also assessed on intracellular amastigote stage of *L. donovani*. The Isobologram analysis for combination of Amphotericin B and MMV688262 was less than 1 (Figure 9). This indicates that the two compounds have synergistic activity.

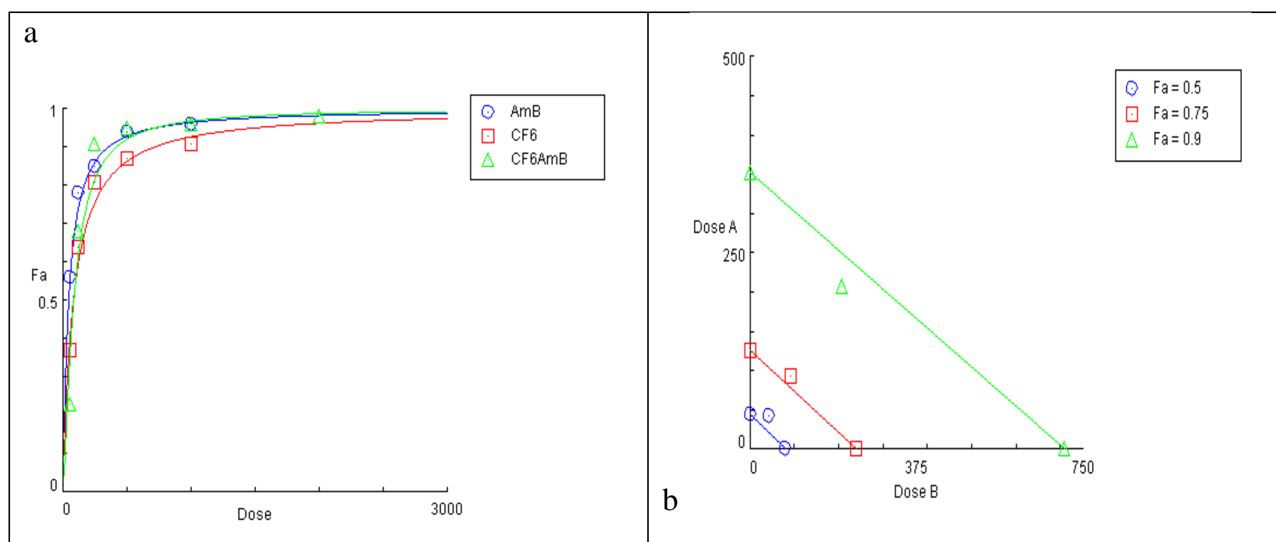

Figure 10: Activity graph: a) Dose-Effect Curve, b) Isobologram for Combination of delamanid with Amphotericin B (CF6AmB [1:1])

5. Discussion

5.1. Promastigotes inhibition assay

5.1.1. Primary screening

Developing new compounds from initial target identification to the final validation usually takes 12- 15 years and costs more than 1 billion dollars (114). These days, repurposing existing drugs or biological compounds especially for NTDs is becoming a normal trend. Drug repurposing is time efficient and cost effective compared to new drug development. Most screening procedures starts with assay development to high throughput screening and goes to hit identification, lead optimization and finally selection of a candidate molecule for clinical development (114,115). Varieties of screening methods exist to identify hit molecules. The high throughput screening (HTS) is one of these methods and involves the screening of entire library in search for a drug target. To this account the present study exploit a medium/high throughput phenotypic screening was done to identify potential 'hits' for lead optimization.

The Pathogen Box contains compounds active against many neglected tropical diseases. These compounds were designed to be tested at a concentration of 1 μM (MMV recommendation). Therefore, all compounds, regardless of their disease set were screened for activity against promastigote stage of *L. aethiopica and L. donovani at* 1 μM. The sole purpose was to generate a complete hit map that would help to select effective compounds for IC_{50} determination. Following the primary screening, all compounds were classified under different range of percentage of inhibition. Active compounds which exhibit > 70% promastigote inhibition were selected for further study.

Among the selected compounds MMV675968, MMV690102 and MMV690103 belong to the pyrimido [4,5-d] pyrimidine-2,4,7-triamine chemotype which are known to inhibit DHFR (Cryptosporidium dihydrofolate reductase). MMV637229, MMV688703, MMV022478 were reported to affect histamine, cGMP dpendent kinase and trypanothionine reductase, respectively. The remaining compounds (MMV687812, MMV663250, MMV024406, MMV011511, MMV024829, MMV085071, and MMV022029) do not have a reported mode of action. Among all selected compounds MMV688262 (Delamanid) was extensively studied for its antileishmanial activity. This compound is a dihydro-nitroimidazooxazole derivative. It acts by

inhibiting the synthesis of mycobacterial cell wall components, methoxy mycolic acid and ketomycolic acid. Since the screen was the first in its kind in *L. aethiopica* species, published resources were scarce. Therefore we couldn't compare our results with other reports. But some compounds anti-leishmanial activities and their mode of actions in leishmaniasis and other disease conditions were reported (Annex 2).

5.1.2. Median inhibitory concentration against promastigotes

All compounds were also produce >70% promastigotes inhibitions with the initial concentration (1µM). This correlates with the result obtained in the primary screening. Almost all compound showed 50% promastigote inhibition at just below 0.5 µM. Especially MMV690102 demonstrated very low IC_{50} (0.010, 0.018) and (0.214, 0.277) in two independent experiments of *L. donovani* and *L. aethiopica* assays. This finding is consistence with the reports of Berry et al. (87) and Duffy et al. (56) which used *L. Mexicana* and *L. donovani* strains, respectively. This indicates the potential of this compound in the treatment of both leishmania forms. The IC_{50} of delamanid was higher than the report of Patterson et al. (116) who reported 0.015 µM on *L. donovani* isolates. This might be resulted from the difference in assay, strain or the amount of HINBCS used. After IC_{50} determination, selected compounds were examined for Cytotoxicity and physico-chemical characteristics to verify their suitability for intracellular amastigotes assay.

5.2. Cytotoxicity of selected Pathogen Box compounds

Selected Pathogen Box compounds were evaluated *in vitro* for their hemolytic activity to sheep RBCs and toxicity to THP-1 cells. All compounds demonstrated good safety at the highest tested concentration (25 µM). However, MMV690102 from the kinetoplastid disease set demonstrated some degree of inhibition. Yet, the extrapolated CC_{50} value was significantly high compared to the IC_{50} value registered on promastigotes. The calculated SI of this compound was more than 700 and 132 for *L. donovani* and *L. aethiopica* strains, respectively. which is greater than the 100 fold selectivity window set for leishmania parasites (83). MMV690102 showed variable cytotoxic effects on different mammalian cells. The calculated SI index of this compound on MRC-5 and PMM cells was found to be lower than the SI determined for THP-1 cells. These variations reflect the need for further studies to outline the effect of this compound on many other types of mammalian cells.

In general, all selected Pathogen Box compounds demonstrated a very good selectivity index with respect to THP-1 cell lines and other mammalian cells and have limited potential to be hemolytic or cytotoxic at therapeutic concentrations.

5.3. Selection of suitable compounds for intracellular amastigote assay

Many compounds and chemical analogues have been synthesized targeting neglected tropical diseases such as leishmaniasis; however most of them failed to progress into clinical trials. Inability to determine a clear mechanism of actions, lack of effective biological targets and a narrow selectivity between the pathogen and the host cells are some of the reason given for these failures (83,114). The Japanese Global Health Innovative Technology (GHIT) in alliance with other stake holders sets a disease‑ specific criteria for hits and leads for malaria, tuberculosis, VL and Chagas disease (83). This disease specific criterion has been used as a guide for lead compound selection in the present study.

Since most of the compounds in the Pathogen Box are pure compounds, they lack enough biological and PK data to support lead compound selection. So we used another approach to select suitable compounds for intracellular amastigotes assay. Compounds suitable for this kind of assay should inherit good cell permeability and absorption as they are required to traverse through two membranes and reach the site of action. Therefore 'Lipinski's rule of five' was used to assess physico-chemical characteristics of selected pathogen box compounds. The rule stated that, poor absorption and/or permeation is more likely when the compound possess more than 5 H-bond donors, 10 H-bond acceptors, the molecular weight (MWT) is greater than 500 and the calculated Log P (CLogP) is greater than 5 (or MlogP 4.15) (83,114,117–119). Based on this rule a table was constructed using physico-chemical data gathered from National Centre for Biotechnology Information (NCBI) and compounds were weighed against the guidelines.

In view of that, MMV687812, MMV688262 (both from TB disease set) were slightly off from the 500 g/mol molecular weight and the 10 hydrogen bond receiver count. However, during drug discovery, lipophilicity and molecular weight are often increased in order to improve the affinity and selectivity of the drug candidate. Hence it is often difficult to maintain drug-likeness (i.e., RO5 compliance) during hit and lead optimization. Therefore, after considering other parameters we reject one compound (MMV687812) and accept the other one for further intracellular

amastigotes assay. All the other compounds fulfilled Lipinski's rules of permeation and absorption. Therefore selection was made based on the percentage of inhibition displayed on the primary screening (inhibition at highest concentration) and the IC_{50} values of each compound (average of the two independent experiments).

As a result among the candidate compounds, MMV085071, MMV688415, MMV690102, MMV022478, MMV022029 and MMV024406 tops other compounds and selected for further studies on intracellular *L. aethiopica* amastigotes assay, while MMV022478, MMV688262, MMV688415 and MMV690102 were selected for intracellular *L. donovani* amastigotes assay.

5.4. Intracellular amastigotes assay

5.4.1. Intracellular amastigotes assay against *L. aethiopica*

The effect of six compounds on intracellular amastigotes was assessed. From these compounds; MMV022029 and MMV024406 were unable to produce IC_{50} at the highest tested concentration (1 μM). Therefore they were replaced by MMV085071 and MMV022478. Of the 6 screened compounds, MMV690102 was found to be a very potent inhibitor of *L. aethiopica* amastigotes. The results obtained for MMV690102 is similar to the finding of Duffy et al. (56) who gathered the data against *L. donovani*. However, the estimated IC_{50} is lower than the report of Berry et al. (67), who reported IC_{50} values of 0.107 μM on *L. mexicana* promastigotes. The difference might be due to species variation or assays type used. Data from MMV indicates a lower IC_{50} value (3 nM) on *L. infantum*, which is 20-fold lower than our finding. Duffy and his colleagues (100) reported that MMV690102 demonstrate inhibitory effect against *L. donovani* without any Cytotoxic activity displayed in the HEK293 cell lines.

MMV085071, MMV022478 and MMV688415 also exhibited good activities on *L. aethiopica* amastigotes. We could not find anti-leishmanial reports on MMV022478, MMV688415 and MMV085071. But MMV688415 was reported to have inhibitory activity on *Trypanosoma Cruzi* (100), whereas MMV022478 was reported to inhibit *Toxoplasma gondii and T. cruzi* (100,106).

MMV022029 and MMV024406 were selected based on their excellent inhibitory activities upon anti-promastigotes assay. These compounds produced 87% (IC_{50}=0.214 μM) and 95% (0.018 μM) inhibition at 1 μM, respectively. Conversely, their activity reduced significantly on the intracellular amastigotes assay. Reduction in activity is expected as the compounds should pass

two membranes to reach amastigotes (120,121). However a very poor activity with significance efficacy difference may suggest a problem on physico-chemical property of the compounds or biological target variation between the two parasite forms. Since these two compounds possessed physico-chemical property desirable for cell penetration, their poor activity against the intracellular stage is more likely attributed to poor stability in the parasitophorous vacuoles (PV). The PV is a phagolysosome-like compartment with acidic and hydrolytic conditions (122) responsible to degrade materials in to low molecular weight components. Therefore, anti-leishmanial drugs are expected to function in two PH ranges (121); lacking this quality may attribute to weak action.

The other factor that may contribute to their poor performance is a structural difference between amastigotes and promastigotes. Inherent differences in transcriptomes and proteosomes of amastigotes and promastigotes may cause difference in susceptibility (123). The cell growth and metabolism difference can be another factor. The free living form is known by its excessive proliferation while the intracellular form is not. The latter have a very slow or technically no growth as compared to the former (123). This biological phenomenon plays a role in drug susceptibility difference between organisms. It is known that actively multiplying organisms are very susceptible to agents that inhibit cell divisions and energy metabolism. Perhaps the major drawback of resazurin based colorometric assay is its inability to differentiate growth inhibitory with cytocidal agents. The extent of reduction of the indicator is greatly depends in cell density; hence low density due to inhibition in cell growth and cell death could give similar result. This limitation often leads to drug efficacy failures in intracellular amastigotes.

5.4.2. Intracellular amastigotes assay against *L. donovani*

Among the top ten compounds identified from the primary screening, four compounds were selected for further intracellular amastigotes inhibition assay on *L. donovani*. From these compounds, MMV690102 and MMV688262 (delamanid) were found to be potent inhibitors. Delamanid and MMV690102 inhibit 50% of amastigote population at 0.053 µM and 0.060 µM, respectively. These values are in comparison with the IC_{50} of Amphotericin B. Therefore, both compounds can be good leads for visceral leishmaniasis.

The current finding on MMV688262 is similar with the report of Patterson et al. (116) who investigate the activity of delamanid against various laboratory and clinical isolates of L. donovani *in vitro* (IC_{50} ranging from 86.5- 259 nM). From which delamanid showed IC_{50} value at 86.5 nM on *L. donovani* isolates obtained from Ethiopia. But our finding is lower than the report of Berry et al. (87) (1.780 µM), this variation might be caused by difference in the assay format (their assay is more sensitive than our assay because the protocol is based on cell viability not parasite burden) or species variation (they used *L. mexicana*). Patterson and his colleagues (116) reported that even strain difference within the same species can lead to a variation in drug susceptibility. The same study also indicates that strain collected from Ethiopia is very susceptible as compared to other strains collected from different parts of the world (116).

5.5. Selected Leads for further drug development

The screening of the Pathogen Box compounds identified two compounds suitable for the two strains. MMV690102 and MMV688262 were identified as the best candidates for *L. aethiopica* and *L. donovani,* respectively. MMV690102 also have got inhibitory activity against *L. donovani*.

5.5.1. MMV690102 (2-N-[(1R)-1-[4-(4-methoxyphenoxy)phenyl]ethyl]-2-N-methyl-pyrimido [4,5-d] pyrimidine -2,5,7- triamine): a lead for *L. aethiopica* and *L. donovani*

The physico-chemical characteristic of this compound makes it a good candidate for further *in vivo* experiments. MMV690102 has got undersized hydrogen receivers and donor counts with six rotatable bond counts (124). It has good lipophilicity and low polar surface area. This compound demonstrated a very good selectivity indexes in various mammalian cells.

MMV690102 was identified from the GSK kinetoplastid screen. In theory, it was believed to be DHFR inhibitor. But a recent study showed that these analogues were found to be ineffective against *malaria* and *T. brucei brucei*. Given that malaria and T. *brucei brucei* are known to be inhibited by DHFR inhibitors, their failure to inhibit these organisms questioned the proposed mechanism of action. Thus the proposed target might be wrong unless the structural divergence between DHFR orthologues expressed in DHFR expressing organisms explained the failure

(56,58,125). In general, our finding pushed us to consider MMV690102 as promising starting points for hit-to-lead optimization for both forms of leishmaniasis.

5.5.2. MMV688262 (Delamanid): a lead for visceral leishmaniasis

Delamanid is a pro-drug similar to pretomanid (PA-824). These two compounds are dihydro-nitroimidazole class, developed to be potent, orally active drug for the treatment of TB (126). It is discovered by Otsuka in a screen for inhibitors of mycolic acid biosynthesis (127). Delamanid went through advanced clinical assessment and has already been approved by the European Medicines Agency and the Japanese Ministry of Health, Welfare and Labour for the treatment of TB (128). Later on, it was found to be effective for the treatment of multi-drug resistance tuberculosis (126). This drug demonstrated excellent bactericidal activity that would make it suitable in regimens designed to shorten treatment duration (126). Its effectiveness was even greater than the reference drug isoniazid (129,130).

Delamanid's activity is not limited on TB only, another study on variety of *L. donovani* strains revealed its potential use in the treatment of leishmaniasis. Delamanid was demonstrated to kill promastigotes in dose dependant manner after 12 hr of treatment, confirming that it is rapidly leishmanicidal (116). Apart from that, it also possesses a very good solubility in aqueous solution. A kinetic solubility assay indicated sufficient aqueous solubility (>250 µM) in 2.5% DMSO (116). This makes it suitable for *in vitro* studies. This compound is very suitable for oral formulation and it was successfully tested in in-vivo (116); which shows the potential of delamanid to be the second orally taken drug for leishmaniasis following miltefosine (83).

Delamanid was demonstrated no effect on CYP1A1/2, CYP2A6, CYP2B6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 (126,130). The oral availability of the drug is greater when administered along with foods as its absorption increases in fed state as compared to fasting state. The peak plasma binding obtained after 4 hrs of administration with high plasma protein binding ($F_u = 0.0045$). The drug has a plasma half life ranging from 30-38 hrs, and its metabolites have >100 hrs. Delamanid showed high volume of distribution of around 1,100 liter after 100 mg twice daily administration. Excretion is mainly via faecal route, urinary excretion is less than 5%. Thus it can be administered for patients with renal impairment and mild hepatic insufficiency. (127,130–132).

The mode of action of delamanid was shown to be inhibition of the synthesis of the mycobacterial cell wall components, methoxy-mycolic and keto-mycolic acid (126,133). Many nitroheterocyclics require bio-activation of their nitro groups to become biologically active (131). In Mycobacterium tuberculosis, delamanid is known to be activated by bioreduction of its nitro group by M. TB enzyme, deazaflavin-dependant nitroreductase (Ddn), known to activate the closely related nitroimidazo-oxazine drug PA-824 (126,131). Given that leishmania complexes are devoid of a Ddn homologue, it was assumed to be activated by the NADH-dependent bacterial-like nitroreductase (NTR) (116,134). However a research done by Patterson and his colleagues (116) revealed that genetically modified promastigotes over expressing NTR, which demonstrated a 13-fold increase in their sensitivity to nifurtimox could not repeat similar degree of sensitivity for delamanid (116). This finding clearly indicates delamanid activation is neither through Ddn nor NTR, Implying a different mechanism of action at play.

5.6. Synergistic effect of lead compounds with reference drugs

The rise of resistant strains in both forms of leishmaniasis is currently one of the talking points in the treatment of leishmaniasis. Resistance to currently used anti-leishmanial drugs have been reported in many parts of the world. Drug resistance reports for currently used anti-leishmanial drugs have been released from Africa, India and Asia for many years. The rate of treatment failures and relapses are also increasing dramatically (59,61).

This indicates the urgent needs of new anti-leishmanial drugs or combination therapy to treat drug resistant leishmanial infections. Treatment with a combination drugs offer better chance to kill resistant pathogens. A pathogen that is mutant to the first drug can be killed by the second drug and vice versa. It is therefore with this account that our study team conduct synergistic effect assessment between lead compounds and conventional medications like Pentamidine and Amphotericin B.

In this study, we were able to identify MMV690102 and MMV688262 (delamanid) as potential hits for *L. aethiopica* and *L. donovani* respectively. These compounds were tested for synergy with commonly used antileishmanial drugs. As it is shown in section (4.3.3), MMV690102 showed antagonistic effect when tested in combination with Pentamidine. But MMV688262 (delamanid) showed synergistic activity with Amphotericin B. This synergistic effect of

delamanid in the inhibition of intracellular amastigote stages of *L. donovani* strains is a very useful observation. The result can be evidence for the potential use of delamanid in drug combination. However, further *in vitro* and *in vivo* studies are required in resistant and sensitive strains to get an insight about its' potential in the development of a treatment for drug resistant leishmaniasis.

6. Conclusion and recommendations

The present study identified a panel of compounds that have activity against the two species of Leishmania *in vitro*. The compounds also appear to be safe. However, good *in vitro* efficacy and safety does not necessarily reflect the *in vivo* situation. Hence, this study should be complimented with *in vivo* studies. Identified compounds can be a good starting point for lead optimization with a broad range of structural diversity as evidenced by the calculated physico-chemical properties (polar surface area; rotatable bonds, H^+ bond donor and receiver and lipophilicity).

Based on the findings of the present study, the following recommendations are forwarded:

1. The screening of Pathogen Box compounds identified 16 hits, from these only 7 compounds were tested in intracellular amastigotes assay. Therefore, the activity of the remaining compounds should be done with conventional, fast and advanced screening techniques.
2. Some compounds have moderate activity against promastigote and strong activity against intracellular amastigotes, therefore additional experiments should be done to investigate the plasma protein binding characteristics of these compounds.
3. Apart from their attractive antileishmanial activities, majority of these compounds lack relevant biological data. Therefore, their PK profile as well as related activities on mammalian cells should be outlined.
4. The performance of MMV022029 and MMV024406 in antiamastigotes assay was significantly decreased. Thus, a structural remodelling should be sought to enhance their intracellular activity.
5. MMV022029 and MMV024406 should be considered for *in vivo* efficacy experiments, Or else, transmission blocking models targeting insect stage should be considered.
6. The two lead compounds (MMV690102 and MMV688262) identified in this study should be progressed to further pre-clinical studies.
7. The antileishmanial mechanism of action of MMV690102 and MMV688262 should be investigated using target based experiments.

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8. Annexes

Annex 1: biological data of active compounds against *Leishmania aethiopica* and *Leishmania donovani* isoltes

Annex 1A: Biological data of selected compounds from malaria diseases set

MMV ID	*P.falci.* 3D7 IC50 (µM)	*P.falc* Dd2 IC50 (µM)	*P. falcip.*W2 IC50 (µM)	*P.berghei* sporozoite 10 µM (inhb %)	*P.berghei* sporozoite IC50 (µM)	*P.falcip* NF54 Gametocytes Stage V: at 10 µM (% inhibition)	P. falcip. NF54 Gametocytes Stage V IC50 (µM)	HepG2 CC20 (µM)[a]	HepG2: inhibition at 10 µM (%)	HepG2 CC50 (µM)
MMV022478	0.8	1.2	1.4	96	>10	97	0.6	0.8		>10
MMV011511	1.2	3.0	4.5	67		4			6	
MMV024829	2.1	5.2	9.3	66	>10	10		13.7		>10
MMV022029	0.8	2.1	2.1	99	4.6	95	1.4	4.2		>10
MMV024406	0.8	1.3	1.6	99	2.3	51		2.7		>10
MMV085071	0.1	0.2	0.2	64	>7.5	12		4.7		>10
MMV663250	1.6	1.5	1.4	83	>10	36		15.2		>10

Annex 1B: Biological data of selected compounds from kinetoplastid diseases set

MMV ID	T.cruzi IC50 (µM)	*T. b. brucei* IC50 (µM)	*T. b. rhodesiense* IC50 (µM)	L. *infantum* (macrophages) IC50 (µM)[a]	MRC5 CC50 (µM)[a]	PMM CC50 (µM)[a]	HepG2 CC20 (µM)[c]
MMV688942	0.06	32.9	28.0	>64	>64	>64	
MMV690102				0.03	5.4	4.0	
MMV690103				0.5	7.1	8.0	
MMV688415	<0.13	16.1	4.0	17.3	>32	>32	38.4

Annex 1C: Biological data of selected compounds from cryptospordia diseases set

MMV ID	Anti - *Cryptosporidium*	Anti - *Cryptosporidium parvum* activity data	Cytotoxicity data	
	IC 50 (µM)[a]	(µM)[b]	HL60 CC50 (µM)	HepG2 CC20 (µM)[c]
MMV675968	4.0	0.1-1.0	>25	3.4

Annex 1D: Biological data of selected compounds from trichuriasis diseases set

MMV ID	Anti - *Ancylostoma ceylanicum* activity data: inhibition (%)[a]	Anti - *Trichuris muris* activity data: inhibition (%)[a]	HepG2 CC20 (µM)[b]	Analogues available
MMV637229	76% at 200 µM (L3) , 47% at 50 µM (adult)	1.5% at 100 µM (L3) , 100% at 50 µM (adult)	9.3	

Annex 1E: Biological data of selected compounds from tuberculosis diseases set

MMV ID	*M. tuberculosis* activity data		*M. tuberculosis* activity data						Cytototoxicity
	Replicati (µM)[a]	Non-repl MIC90 (µM)[a]	GAST/Fe MIC (µM)	7H9/ADC/Tw MIC (µM)	7H9/ADC/Tw MIC (µM)	7H9/cholesterol/BSA/Tx MIC (µM)	7H9 /Tx MIC (µM)	7H9/2.5mM MIC90 (µM)	HepG2 CC50 (uM)[a]
MMV688262	0.08	0.2	0.1	0.1	0.2	0.05	0.6	12.5	72.5
MMV687812	3.5	3.1	1.6	1.2	1.6	0.4	0.6	1.5	3.9

Annex 1F: biological data of selected compounds from toxoplasmosis diseases set

	Anti - Toxoplasma gondii activity data							Cytotoxicity
MMV ID	Inhibition of plaque forming (%),	Inhibition of plaque forming efficiency (%),	2-day killing assay: inhibition at 10 µM (%), Experiment C	2-day killing assay: inhibition at 10 µM (%), Experiment D	2-day killing assay: inhibition at 10 µM (%), Experiment E	2-day killing assay: IC50 (µM), Experiment F	2-day killing assay: IC50 (µM), Experiment G	HL 60 CC50 (µM)
MMV688703	100		99	100	98	1.2		>50

Annexes 2: Summary of disease set, known activities and mode of actions of selected compounds

MMV ID	Disease set	Activity on this assay	Known activity	Mode of action	References
MMV675968	Cryptosporidiosis	L.*donovani*	T.gondii, P.falciparum, C.albicans	inhibitors of DHFR	(56,58,135)
MMV688942	Kinetoplastids	L.d & *L*.aet	T.cruzi	CYP51 or sterol biosynthesis activity	(56)
MMV690102	Kinetoplastids	*L*.d & *L*.aet	*L.donovani*, *L.mexicana*	inhibitors of DHFR	(56,87)
MMV690103	Kinetoplastids	*L.donovani*	*L.donovani*	inhibitors of DHFR	(56)
MMV688415	Kinetoplastids	*L*.d & *L*.aet			
MMV022478	Malaria	*L*.d & *L*.aet	T. gondii	trypanothionine reductase, inhibit NADPH oxidase 4	(56,58)
MMV011511	Malaria	*L.donovani*	-	-	
MMV024829	Malaria	*L.aethiopica*	-	-	
MMV022029	Malaria	*L.aethiopica*	T.brucei brucei	-	(56)
MMV024406	Malaria	*L.aethiopica*	C. albicans,	-	(135)
MMV085071	Malaria	*L.aethiopica*	-	-	
MMV663250	Malaria	*L.aethiopica*	-	-	
MMV688703	Toxoplasmosis	*L.donovani*	P.falciparum, T. gondii	inhibitor of cGMP-dependent protein kinase	(56,58)
MMV637229	Trichuriasis	*L*.d & *L*.aet	T.cruzi,	Antihistamine	(56)
MMV688262	Tuberculosis	*L.donovani*	Tuberculosis, *L.donovani*	mycobacterial cell wall	(83,87,116,127, 131,132)
MMV687812	Tuberculosis	*L.aethiopica*	-	-	

Description: DHFR= Cryptosporidium dihydrofolate reductase, L.d & L.aet=L.donovani and L. aethiopica

Annex 3: Preparation NNN media and Locks solution

Preparation of NNN medium

9.2g Nutrient agar, 0.6g D-(+) Glucose (anhydrous) and 2.4 g sodium chloride were weighed, mixed and dissolved in 400ml of distilled water by boiling in hot plate with repeated shaking until clear solution obtained. Then it was autoclaved at 121^{0}C for 30 minutes. Rabbit or Sheep blood priorly collected with a sterile bottle containing glass beads was defibrinated by shaking and heat inactivated by keeping it in 37 ^{0}C water bath for 50 minutes and then transformed into 56 ^{0}C water bath for 20 minutes. The autoclaved ingredients (400 ml) and of heat inactivated blood (100 ml) were mixed at 50 ^{0}C. 2ml of the mix is dispensed among culture vials, allowed to settle slant and stored at 4 ^{0}C until use.

Preparation of Locke's solution

The ingredients 4.5g sodium chloride ,0.2g potassium chloride, 0.1g calcium chloride ,0.1g sodium bicarbonate and 1.25g D-(+)-glucose(anhydrous) all from sigma were mixed and dissolved in 500ml distilled water. The mixture was autoclaved at 121 ^{0}C for 30 minutes and stored at 4 ^{0}C until use.