# OPTIMISATION OF *IN VITRO* INTRACELLULAR ANTILEISHMANIAL ASSAY AND EVALUATION OF *Senna spectabilis* (DC.) H. S. IRWIN BARNEBY METHANOLIC LEAF EXTRACT AND ITS CONSTITUENTS AGAINST *Leishmania major* USING BIOASSAY-GUIDED ISOLATION APPROACH

by

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| | |
|---|---|
| % | Percent or percentage |
| $[M+M]^+$, *m/z* | Molecular ion peak in mass spectrometry |
| $[\alpha]_D^{20}$ | Optical rotation at temperature 20 degree Celsius |
| < | Maths symbol for 'lesser than' |
| = | Maths symbol for 'equals to' |
| > | Maths symbol for 'greater than' |
| ± | Maths symbol for 'plus or minus' which indicates the range of a value |
| °C | Degree celsius |
| µg | Micrograms |
| µL | Microlitre |
| 10x, 20x, 40x, 100x | Scale of objective lens (nose piece) for microscopical field observation |
| $^{13}$C NMR / CMR | Nuclear magnetic resonance spectroscopy to carbon |
| $^{1}$H NMR / PMR | Nuclear magnetic resonance spectroscopy to hydrogen |
| $^{1}$H-$^{1}$H COSY | Homonuclear correlation in nuclear magnetic resonance |
| 2D NMR | Two dimensional nuclear magnetic resonance |
| 3R | Replacement, reduction and refinement |
| ABSL II | Animal biosafety laboratory class II |
| Amp-B | Amphotericin B |
| ATCC | American Type Culture Collection |
| BALB/c mice | Albino, laboratory-bred strain of the house mouse |
| bd | Broad |
| BSC II | Biosafety cabinet class II |
| BSL II | Biosafety laboratory class II |
| CC | Column chromatography |
| CDC | Centers for Disease Control |
| $CDCl_3$ | Deutero chloroform |
| cells/mL | Cell number per milliliter |
| $CH_2$ | Methylene |
| CL | Cutananeous leishmaniasis (also known as localized CL, LCL) |

| | |
|---|---|
| $cm^{-1}$ | Reciprocal cm. Unit for stretching frequency (wave numbers) in mass spectrometry |
| $COCH_3$ | Acetic acid radical |
| $conc^{1,2,3,4}$ | Concentration labeling (concentration 1,2,3 & 4) |
| COSY | Correlation spectroscopy |
| CS- | *Senna spectabilis* parts, fractions or compound |
| DCL | Diffused cutaneous leishmaniasis |
| DEPT | Distortionless enhancement by polarization transfer |
| DMSO | Dimethyl sulfoxide reagent |
| DNA | Deoxyribonucleic acid |
| DND*i* | Drug Neglected Diseases Initiative |
| $EC_{50}$ | The half maximal effective concentration |
| EtOAC | Ethyl acetate |
| F | Fraction (with number) |
| FBS | Fetal bovine serum |
| g | Grams, unit for mass |
| GLP | Good laboratory practice |
| H | Hydrogen |
| HAT | Human African trypanosomiasis |
| HIV/AIDS | Human immunodeficiency virus infection / acquired immunodeficiency syndrome |
| HMBC | Heteronuclear multiple bond correlation |
| HMQC | Heteronuclear multiple-quantum correlation |
| Hz | Hertz, unit of frequency |
| $IC_{50}$ | The half maximal inhibitory concentration |
| IPARF | IPharm animal research facility |
| ATR-IR | Infrared radiation (IR) using attenuated total reflectance (ATR) |
| *J* | Coupling constant |
| *L. major* | *Leishmania major* |
| $Log_{10}$ | Logarithm to base 10 |

| | |
|---|---|
| LSHTM | London School of Hygiene and Tropical Medicine, The University of London, United Kingdom |
| m (in $^{13}$C NMR) | Multiplet |
| M | Molarity, unit for concentration |
| MCL | Mucocutaneous leishmaniasis |
| -Me, $-CH_3$ | Methyl group |
| MeOH | Methanol |
| mg | Milligrams |
| MHz | Mega Hertz |
| mL | Milliliter |
| mp | Melting point |
| MS | Mass spectrum |
| *n*-BuOH | *n*-Butanol |
| ND | Not determined |
| ng/mL | Nanograms per milliliter |
| NGOs | Non-government organizations |
| *n*-Hex | *n*-Hexane |
| nM | Nanomolar |
| -OH | Hydroxyl group |
| PCR | Polymerase chain reaction |
| PDT | Population doubling time |
| PEM | Peritoneal exudates macrophages |
| Penstrep | Penicillin/ streptomycin |
| PKDL | Post-Kala-Azar dermal leishmaniasis |
| PMA | Phorbol 12-myristate 13-acetate |
| PPE | Protective personnel equipment |
| ppm | Parts per-million |
| q | Quartet |
| R&D | Research and development |
| $R_f$ | Retardation factor value |
| rpm | Rotation per minute |

| | |
|---|---|
| s | Singlet |
| SI | Selectivity index |
| SPF | Specific pathogen free |
| t | Triplet |
| TLC | Thin layer chromatography |
| VL | Visceral leishmaniasis |
| VLC | Vacuum liquid chromatography |
| WHO | World Health Organization |
| δ | Delta chemical shift |
| $\nu_{max}$ | Maximum wavelength |

**PENGOPTIMAAN ASAI ANTILEISHMANIA INTRASEL *IN VITRO* DAN PENILAIAN EKSTRAK METANOL SERTA KOMPONEN-KOMPONENNYA DARIPADA DAUN *Senna spectabilis* (DC.) H. S. IRWIN BARNEBY TERHADAP *Leishmania major* MELALUI PENDEKATAN PENGASINGAN BERPANDUKAN BIOASAI**

## ABSTRAK

Setiap tahun, berjuta-juta manusia dijangkiti penyakit leishmaniasis. Pendekatan rawatan alternatif diperlukan kerana majoriti pesakit tersebut menjalani kehidupan di negara-negara yang tidak mampu dari segi kewangan dalam penemuan drug berpacukan pasaran. Rawatan sedia ada berhadapan dengan pelbagai kesan sampingan, seperti ketoksikan jantung dan ketoksikan ginjal pesakit, dan tempoh rawatan panjang yang boleh menyebabkan kerintangan parasit. *Senna* spp telah pun dikenali sebagai tumbuhan yang mempunyai kesan antiparasit dan baru-baru ini dilaporkan mempunyai aktiviti antileishmania terhadap *L. major* ekstrasel. Dalam projek ini, dua asai telah digunakan untuk menguji aktiviti antileishmania secara *in vitro* iaitu asai *leishmanicidal* dan asai amastigot intrasel. Tujuan utama kajian ini adalah untuk mewujudkan dan mengoptimumkan asai amastigot intrasel *in vitro* di samping mengekalkan asai *leishmanicidal* sebagai kaedah saringan awal bagi mendapatkan calon drug. Asai amastigot intrasel yang telah dioptimumkan telah digunakan untuk menilai aktiviti antileishmania komponen-komponen aktif tumbuhan yang mempunyai nilai-nilai perubatan iaitu *S. spectabilis* melalui pendekatan pengasingan berpandukan bioasai. Pada mulanya, penilaian aktiviti antileishmania daripada ekstrak metanol daun *S. spectabilis* telah dijalankan menggunakan asai *leishmanicidal* yang juga dikenali sebagai asai promastigot ekstrasel *in vitro*. Asai tersebut menggunakan parasit *L. major* berfasa promastigot. Asai ini dijalankan berdasarkan asai Alamar Blue® dengan sedikit pengubahsuaian. Hasilnya, ekstrak etil asetat (CS-EA) *S. spectabilis* didapati aktif dengan nilai $IC_{50}$ = 70.29 ± 0.38 μg/mL yang kemudiannya telah difraksinasikan. Untuk penilaian antileishmania seterusnya, asai amastigot intrasel *in vitro* telah digunakan, dan asai ini merupakan yang pertama dibangunkan di Malaysia. Dalam asai ini, *L. major* berfasa amastigot telah digunakan untuk menginfeksi sel THP-1 (makrofaj) secara stabil sebelum infeksi tersebut dirawat dengan komponen-komponen daripada *S. spectabilis* menggunakan format slaid 16-takungan ruang kultur sel. Bilangan pembenihan sel-sel THP-1 telah dioptimumkan pada

$2.0x10^4$ sel/takungan dan nisbah infeksi telah dioptimumkan pada 5: 1 (parasit kepada sel perumah). Asai tersebut kemudiannya disahkan menggunakan Amp-B sebagai drug piawai dengan nilai $EC_{50}$ = 0.437 ± 1.06 μM. Seterusnya, pengasingan berpandukan bioasai dilakukan yang mana ekstrak dan fraksi telah diuji dengan asai antileishmania. Ekstrak dan fraksi yang menujukkan aktiviti tertinggi telah dijalankan proses pengasingan kimia lanjutan dan melalui pelbagai prosedur penulenan seperti kromatografi kolum (CC). Proses fraksinasi kromatografik telah diulang sehingga sebatian aktif diperoleh. Pendekatan pengasingan berpandukan bioasai dalam penyelidikan ini menyebabkan fraksinasi lanjut ke atas ekstrak etil asetat melalui satu siri proses kromatografi yang melibatkan VLC, CC dan *flash* CC yang akhirnya menghasilkan komponen bioaktif terhadap *L. major* dengan nilai $EC_{50}$ = 20.584 ± 1.65 μg/mL . Berdasarkan data yang diperoleh daripada analisis spektroskopi terutamanya spektrometri jisim (MS), spektrum inframerah (IR) dan resonans magnetik nuklear (NMR), komponen bioaktif tersebut telah ditentukan sebagai sebatian alkaloid piperidina, (+)-spektalina. Produk semula jadi yang diperoleh ini mempinyai nilai sitotoksik $IC_{50}$ = 45.57 μg/mL atau 0.14 ± 1.06 μM dan nilai indeks selektiviti, SI = 2.22. Projek ini telah disimpulkan dengan pembangunan dan pengoptimaan asai antileishmania intrasel *in vitro*, diikuti dengan pengasingan sebatian dari *S. spectabilis* yang telah dikenalpasti sebagai (+)-spektalina, aktif terhadap infeksi *in vitro* melalui pendekatan pengasingan berpandukan bioasai. Hasil daripada projek ini diharapkan dapat membantu usaha-usaha pembangunan drug untuk merawat penyakit leishmaniasis.

# OPTIMISATION OF *IN VITRO* INTRACELLULAR ANTILEISHMANIAL ASSAY AND EVALUATION OF *Senna spectabilis* (DC.) H. S. IRWIN BARNEBY METHANOLIC LEAF EXTRACT AND ITS CONSTITUENTS AGAINST *Leishmania major* USING BIOASSAY-GUIDED ISOLATION APPROACH

## ABSTRACT

Leishmaniasis affects millions of people each year. Alternative approaches are needed because majority of those infected live in countries which could not afford financially in market-driven drug discovery. Current treatments faced side effects such as cardiotoxicity and nephrotoxocity, and require a long course of treatment which may develop resistance of parasites. *Senna* spp have been known for antiparasitic characteristics and recently reported for its antileishmanial activity against extracellular *L. major*. There are two assays used to test *in vitro* antileishmanial activities in this work which are leishmanicidal and intracellular amastigote assays. The main aim of this study was to establish and optimise the *in vitro* antileishmanial intracellular amastigote assay, while maintained the leishmanicidal assay as the preliminary screening tool to obtain the hits. The optimised intracellular amastigote assay was then used to evaluate antileishmanial activities of active constituents from a medicinal plant, *S. spectabilis* via bioassay-guided isolation approach. At first, the evaluation of antileishmanial activity of methanolic leaves extract from *S. spectabilis* were carried out using the leishmanicidal assay which was also known as *in vitro* extracellular promastigote assay with promastigote stage of *L. major* as the parasite. This assay was done based on Alamar Blue® assay with little modification. As a result, ethyl acetate (CS-EA) extract of the plant was found to be active with $IC_{50}$ = 70.29 ± 0.38 µg/mL and was further fractionated. For further antileishmanial evaluation of fractions, the *in vitro* intracellular amastigote assay was used, and this assay was first to be established in Malaysia. In this assay, amastigote stage of *L. major* was used to infect THP-1 cell (macrophage) stably before treating the infection with substituents from *S. spectabilis* in 16-well chamber slide format. The seeding number of THP-1 cells was optimized at $2.0x10^4$ cells/well and ratio of infection was optimised at 5:1 of parasite to host. The assay was then validated using Amp-B as the standard drug with $EC_{50}$ = 0.437 ± 1.06 µM. Next, bioassay-guided isolation took place where extracts or partitions were subjected to antileishmanial assay and those exhibited

potential activity was carried forward for further isolation and various procedures of purification such as column chromatography (CC). The process of chromatographic fractionation was repeated until an active compound was obtained. Bioassay-guided isolation approach in this work resulted in further fractionation of the ethyl acetate extract with a series of chromatography processes which involved VLC, CC and flash CC which finally yielded the bioactive constituent against *L. major* with $EC_{50}$ = 20.584 ± 1.65 µg/mL. The bioactive constituent was determined as piperidine alkaloid named (+)-spectaline based on the spectroscopic analysis mainly mass spectrometry (MS), infrared spectral (IR) and nuclear magnetic resonance (NMR). This natural compound showed cytotoxicity $IC_{50}$ = 45.57 µg/mL or 0.14 ± 1.06 µM and selectivity index, SI = 2.22. This work concluded with assay development and optimisation of *in vitro* antileishmanial intracellular assay, followed by isolation of a compound from *S. spectabilis* which was identified as (+)-spectaline, active against the *in vitro L. major* infection via bioassay-guided isolation approach. The finding from this work hopefully will aid the drug development efforts for leishmaniasis treatment.

# CHAPTER ONE

## INTRODUCTION

Neglected diseases are a group of tropical infections that affecting human populations in mainly in low-income countries. Those diseases are leading cause of mortality, chronic disability and poverty (Pedrique *et al.*, 2013). Human African Trypanosomiasis (HAT) or most commonly known as sleeping sickness, leishmaniasis, and Chagas' disease are considered the most neglected because received less media attention and research funding as compared to human immunodeficiency virus infection or acquired immunodeficiency syndrome (HIV/AIDS), tuberculosis, and malaria (WHO, 2015). Regmi and colleagues (2014) reviewed that overall research and development (R&D) funding has increased by more than 70% for the core neglected tropical diseases (NTDs), however it is not correlated with disease burden or attributed deaths and, efforts to tackle these diseases does not seem to be aligned with need.

In 2013, Pedrique and colleagues summarized that there was only 5 out of 420 or 1% new therapeutics approved from 2000 to 2011 are for neglected tropical diseases (NTDs). In addition, approximately 0.1% of global investment in health research is devoted to drug discovery for selected tropical diseases, including leishmaniasis, trypanosomiasis and malaria (Pink *et al.*, 2005). In other words, the world is scarce of drugs for NTDs.

Drugs for Neglected Diseases Initiative (DND*i*), an independent association that is developing new treatments for neglected diseases is a non-profit drug R&D organisation. Private industries, public institutions, academia and non-government

organizations (NGOs) are the collaborators in building this large R&D portfolio for the neglected diseases. The Pan-Asian Screening Network for Drugs for Neglected Diseases from Natural Substances (PAN4ND) is one of the deliverables during DND*i*'s first annual meeting in Tokyo in May 2006. The DND*i* network is centralized at Geneva, Switzerland and the formation was funded by a generous contribution from the Sasakawa Peace Foundation, Japan. The funds as well as collaborations were given and made among countries such as India, South Korea, Japan, Singapore, China, Australia and Malaysia. Since the establishment of DND*i* in 2003, the organization has continued to develop new drugs or new formulations of existing drugs for patients suffering from those diseases (Pink *et al.*, 2005; "About Us – DND*i*" 2013).

Leishmaniasis is a vector-borne obligate intracellular protozoan infection whose clinical spectrum, depends largely both on parasite species and host immune response. The disease ranges from asymptomatic infection to three main clinical syndromes which are visceral leishmaniasis (VL) or known as 'kala- azar', cutaneous leishmaniasis (CL), and mucosal or mucocutaneous leishmaniasis (MCL) also known as 'espundia' (Mansueto *et al.,* 2014). Leishmania infections typically originate from the bite of sandflies belonging to either *Phlebotomus* spp (Old World) or *Lutzomyia* spp (New World), which transmit the parasites into the skin of the host (Mears *et al.,* 2015). Currently, leishmaniasis is facing parasitic resistance against generic pentavalent antimonials which is the first line therapy. Besides, amphotericin B and miltefosine which are the alternative or second line treatment are being effectively used but their high cost and therapeutic complications limit their use in endemic areas. Furthermore, the absence of a vaccine candidate and lack of synthetic chemistry resulted in limited

drug options. Thus, identification and characterization of novel drugs and targets is a major requirement of leishmanial research, especially the ones involved natural products (Singh *et al.*, 2014).

Plants are undeniably a source of medicine and alternative sources of chemotherapeutic agents (Ode *et al.*, 2011). Unfortunately, natural extracts are often extremely complex and contain many unknown compounds (Weller, 2012). These components can clarify structure-activity relationship (SAR) between the basic component of extract and the biological molecule (Koehn and Carter, 2005). In this situation, the use of bioactivity-related analytical or bioassay-guided isolation is helpful. Isolation of the biologically 'active' compound is needed to aid in pharmacognosy, particularly in studies of biomolecular interactions, SAR, toxicity and chemical synthesis of the drug candidates (Weller, 2012).

## 1.1 Problem Statement

1.1.1 Although leishmaniasis is not an issue in Malaysia, Malaysia with 6 other countries committed in joining a consortium called The PAN4ND. The establishment of this consortium is to help DND*i* in finding alternative treatment or searching for new drugs for neglected diseases by creating a climate suitable for collaborative research on identification of novel molecules that could be used for neglected diseases, and to provide the partner institutions with information regarding neglected diseases' research, natural products, and biological screening (www.pan4nd.org).

1.1.2 At present, there is no vaccine exist either for visceral leishmaniasis and cutaneous leishmaniasis. Available drugs are facing resistance, causing

organs toxicity and drawbacks including requirement of close monitoring of patients, need for hospitalization, long treatment duration and other adverse effects.

1.1.3 Our research team has been engaged in the intense search for new natural products for treatment of leishmaniasis from plants and other biodiversity resources. As much as 125 plant extracts were screened against *L. major* promastigotes and 4 were found to be active. One of the active plant namely *S. spectabilis* was selected for further study via bioassay-guided isolation approach in searching of a compound that is active in reducing infection of *L. major* amastigotes parasite on human macrophages *in vitro*.

## 1.2 Justification for selecting *Senna spectabilis*

Parasite that causes leishmania is called as kinetoplastid protozoa or kinetoplastids. Kinetoplastids are flagellated protozoans, which are unicellular eukaryotic organisms and responsible for serious human diseases, such as leishmaniasis, trypanosomiasis and Chagas disease (Pena *et al.*, 2015). All kinetoplastids share a unique mitochondrial DNA structure called kinetoplast DNA, or kDNA in their single large mitochondrion. Besides, they have similar genomic organization and cellular structures such as a single flagellum for motion, and glycosomes as well as kinases, protesases and cytochromes (Ibrahim *et al.*, 2011; Stuart *et al.*, 2008). These similarities may increase the chances of obtaining positive activities for both antitrypanosoma and antileishmania on common compounds. This has been proven in a few screening programs against

kinetoplastids (Pena *et al.*, 2015; Ibrahim *et al.*, 2011) such as in a research of Annang and colleagues 2014 that found a compound known as bafilomycin B1 active against leishmania and trypanosoma parasites. *S. spectabilis* leaves extract was reported to have moderate antitrypanosomal activity by our research group (Zahari, 2014), and due to wide spectrum of biological and pharmacological properties described for *S. spectabilis* and mainly for antitrypanosomal activity reported earlier, this plant was included in an on-going bioprospection project searching for new natural resources and secondary metabolites with antileishmanial activity as goal.

## 1.3 Aim of the study

In order to evaluate the antileishmanial properties of constituents from *S. spectabilis* plant, reproducibility and reliability of results in assays are very crucial. Thus, optimisation and validation of the *in vitro* antileishmananial intracellular amastigote assay are needed through objectives as below:-

1.3.1 To optimise the *in vitro* antileishmanial intracellular amastigote assay through the isolation of parasites (*L. major*) from animals (BALB/c mice) and growth profile studies of *L. major* promastigotes and macrophages (THP-1 cell line).

1.3.2 To validate the *in vitro* antileishmanial intracellular amastigote assay with at least two standard drugs, hence evaluating the antileishmanial property of constituents from *S. spectabilis* leaves by using bioassay-guided isolation approach.

1.3.3 To isolate, elucidate and identify the most active constituent from *S. spectabilis* leaves based on *in vitro* antileishmanial intracellular amastigote assay evaluation.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Leishmaniasis

Leishmaniasis is a group of infectious diseases caused by protozoan parasites. Protozoans are single-celled eukaryotic organisms with nuclei that show some unique characteristics and are usually associated with animals. Their origin is of the genus *Leishmania* in the family Trypanosomatidae and they are well known as kinetoplastids. Kinetoplastids are a group of flagellated protozoa, including the trypanosomatids, that are distinguished by the presence of kinetoplast, a DNA-containing granule located within the single mitochondrion and associated with the flagellar bases (Pink *et al.*, 2005; Cox, 1993).

Leishmaniasis is considered as one of the most NTDs in the world (Seifert, 2011; Eissa *et al.*, 2011; Tiuman *et al.*, 2011). It is a poverty-related disease as it affects the poor in the less developed world (Murray *et al.*, 2001; Trouiller *et al.*, 2002) and is associated with malnutrition (Rocha *et al.*, 2005), displacement, poor housing, illiteracy, weakness of the immune system and lack of resources. Other than that, it is also linked to environmental changes such as deforestation and urbanization which then becomes a serious impediment to socioeconomic development ("Leishmaniasis" 2013).

The diseases are characterized by a spectrum of clinical manifestations which are visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) and rarer manifestations such as mucosal leishmaniasis (or MCL) and post-kala-azar dermal leishmaniasis (PKDL) (Seifert, 2011; Reithinger *et al.*, 2007). There are an estimated 350 million people at risk of infection and disease with 12 million people infected world wide by the

year of 2014 (da Silva *et al.*, 2014). It is endemic in 88 countries around the world with 1.5 to 2.0 million new cases and mortality rate of 70,000 each year (Eissa *et al.*, 2011).

The majority of leishmania infections are zoonotic in origin (Reithinger *et al.*, 2007), though there are important circumstances where man-to-man transmission has developed either transiently or long term, sometimes with devastating results. The actual outcome of a given infection depends on the species or strain of parasite, immune status of the host and the host's immunological competence. There is no absolute difference between parasites causing VL or CL in man. Therefore, strains which have high affinity to infect skin may extend to the lymph nodes, and even visceralise, causing VL (Molyneux and Ashford, 1983).

### 2.1.1 Cutaneous leishmaniasis (CL) and *Leishmania major*

CL or localized cutaneous leishmaniasis LCL, the most common form of leishmaniasis, is a group of diseases with a varied spectrum of clinical manifestation, which range from small cutaneous nodules to gross mucosal destruction (Tiuman *et al.*, 2011; Reithinger *et al.*, 2007). There are some CL species which metastasise to other skin sites especially when cell mediated immunity fails, causing diffuse cutaneous leishmaniasis (DCL) and some metastasize to oronasal tissues causing mucocutaneous leishmaniasis (MCL) (Croft and Yardley, 2002). Table 2.1 shows the CL species with their clinical pathology and distributions.

Table 2.1 : Species of leishmania that cause human disease, with main clinical pathology and geographical distributions. Table is adopted from Reithinger *et al* (2007).

| | Main Clinical Pathology | Main Geographical Distributions |
|---|---|---|
| New World *Leishmania* spp:- | | |
| *L (Viannia) braziliensis* | LCL, mucosal | South America, parts of Central America, Mexico |
| *L (Viannia) panamensis* | LCL, mucosal | Northern South America and southern Central America |
| *L (Viannia) peruviana* | LCL | Peru |
| *L (Viannia) guyanensis* | LCL | South America |
| *L (Viannia) lainsoni* | LCL | South America |
| *L (Viannia) colombiensis* | LCL | Northern South America |
| *L (Leishmania) amazonensis* | LCL, DCL | South America |
| *L (Leishmania) mexicana* | LCL, DCL | Central America, Mexico, USA |
| *L (Leishmania) pifanoi* | LCL | South America |
| *L (Leishmania) venezuelensis* | LCL | Northern South America |
| *L (Leishmania) garnhami* | LCL | South America |
| Old World *Leishmania* spp:- | | |
| *L (Leishmania) aethiopica* | LCL, DCL | Ethiopia, Kenya |
| *L (Leishmania) killicki* | LCL | North Africa |
| *L (Leishmania) major* | LCL | Central Asia, north Africa, middle east, East Africa |
| *L (Leishmania) tropica* | LCL | Central Asia, middle east, parts of north Africa, southeast Asia |
| *L (Leishmania) donovani* | Visceral, LCL | Africa, central Asia, southeast Asia |
| Old and New World *Leishmania* spp:- | | |
| *L (Leishmania) infantum* | Visceral, LCL | Europe, north Africa, Central America, South America |

'New World' refers to the Western Hemisphere while 'Old World' refers mainly to the Eastern Hemisphere. As for the vectors, there are approximately 30 species or subspecies of sandflies with more than 40 additional species probably involved in the parasite transmission (Killick-Kendrick, 1999; Reithinger *et al.*, 2007).

Each year, the number of new cases of VL worldwide is thought to be about 500,000 while those for CL are approximately 1.5 million (Eissa *et al.*, 2011). CL is endemic in more than 70 countries worldwide, and 90% of cases occur in Afghanistan, Algeria, Brazil, Pakistan, Peru, Saudi Arabia and Syria (Figure 2.1) (Desjeux, 2004; Reithinger *et al.*, 2007). The number of cases has increased worldwide during the past decade, as documented in Afghanistan, Bolivia, Brazil, Colombia, Peru and Syria (Reithinger *et al.*, 2007). Most of the infections are symptomless or misdiagnosed (Escobar *et al.*, 1992), causing the global burden of CL being likely to be underestimated (Reithinger *et al.*, 2007).

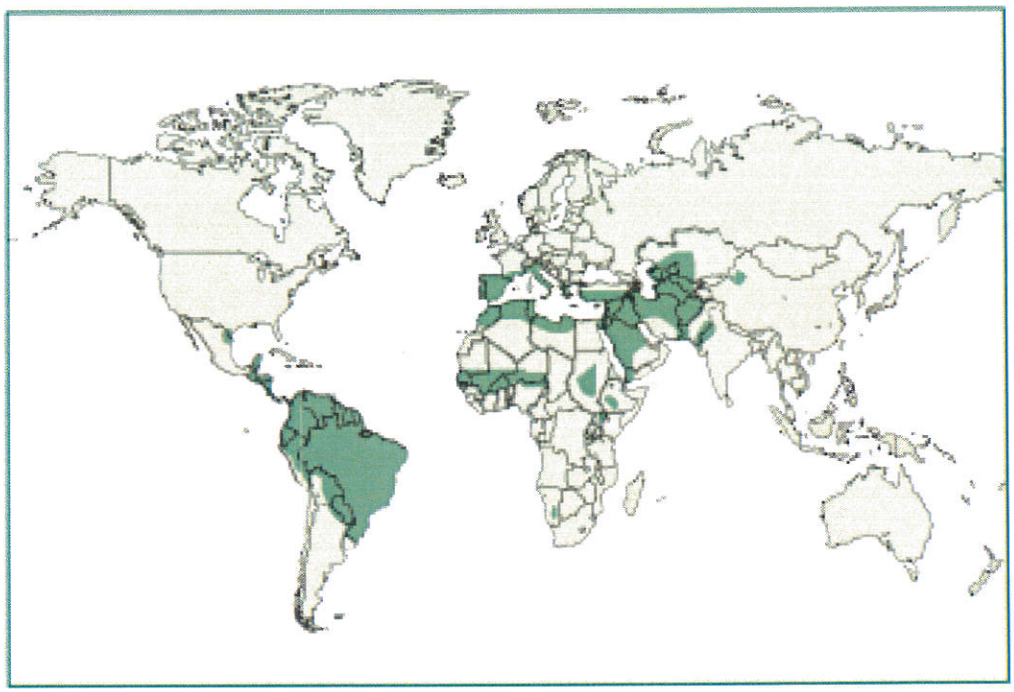

Figure 2.1: The geographical distribution of CL was marked with green (Reithinger *et al.*, 2007).

The taxonomic position of parasite studied is as in Table 2.2. Protozoa are unicellular eukaryotic cells measuring 1-150 μm with metabolic pathways akin to those of the host. This characteristic allows them to evade host immune responses thus surviving in the host (Cox, 1993). The most studied genu of the family Trypanosomatidae from the order of Kinetoplastida are *Leishmania* and *Trypanosoma*, which are also known as kinetoplastids flagellates, trypanosomatids or kinetoplastids (Molyneux and Ashford, 1983). *Leishmania* spp has a single locomotory flagellum rising from a flagellar pocket. It has a unique structure which is diagnostic feature of the order of Kinetoplastida that compact with DNA of mitochondrion genome named kinetoplast. Kinetoplast situated near the base of the flagellum, which appeares as a rod shape when stained (Figure 2.2) (Molyneux and Ashford, 1983; Cox, 1993; Chan-Bacab and Pena-Rodriguez, 2001).

The differences between four common forms of leishmania parasite or kinetoplastids were illustrated in Figure 2.2. The kinetoplast for trypomastigote (A) is located posterior to the nucleus, the flagellum emerges onto the surface near the kinetoplast and attached to the cell body along most of its length. Whereas, the kinetoplast for epimastigote (B) is located anterior to the nucleus, the flagellum emerges onto the surface near the kinetoplast, and its proximal part is attached to the cell body. On the other hands for promastigote (C), the kinetoplast is located anterior to the nucleus and close to the anterior end of the cell. The majority of the flagellum is not attached to the cell body. The amastigote (D) conversely, kinetoplast is located anterior to the nucleus and close to the anterior end of the cell. The short flagellum does not emerge onto the cell surface but is entirely located in the flagellar pocket (Hayes *et al.*, 2014).

Table 2.2: Taxonomic position of *Leishmania major*. Based on Shaw (1994).

| | |
|---|---|
| Kingdom | Protista |
| Subkingdom | Protozoa |
| Phylum | Sarcomastigophora |
| Subphylum | Mastigophora |
| Class | Zoomastigophora |
| Order | Kinetoplastida |
| Suborder | Trypanosomatina |
| Family | Trypanosomatidae |
| Genera | ***Leishmania*** |
| Sub-genera | ***Leishmania*** |
| Species (example) | ***L (Leishmania) major*** |

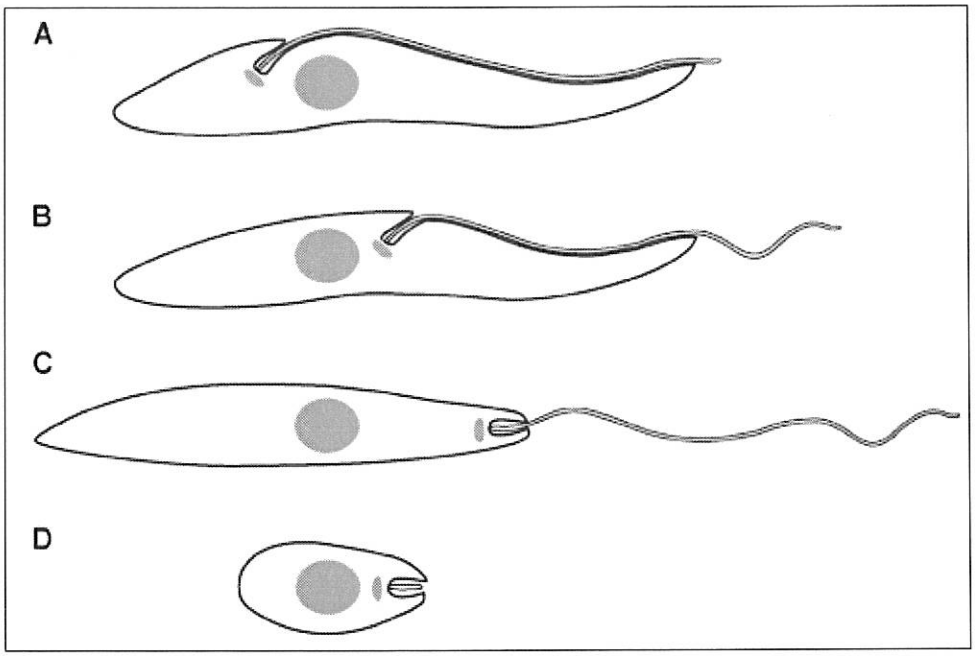


Figure 2.2: Various forms of kinetoplastid flagellates in their life cycle. (A) trypomastigote, (B) epimastigote, (C) promastigote and (D) amastigote. Figure was adopted from Hayes *et al.* (2014).

*Leishmania major* is the first *Leishmania* spp which has been fully sequenced with a genome size of 32.8Mb in 36 chromosomes (Ivens *et al.*, 2005). It is commonly used in *in vitro* and in animal models in antiparasite drug discovery studies (Pink *et al.*, 2005). *L. major* is one of the species that causes CL in Asia, Middle East and North Africa or the Old World (Ngan, 2005). It also causes visceral intracellular protozoal infection. Cultivation of most of the Old World strains is easier than *L. braziliensis* (New World - The Western Hemisphere). Different sandflies carry specific *Leishmania* spp, and mostly *L. major* is carried by the female *Phlebotomine papatasi* sandfly (Figure 2.3), while gerbils, small mammal of order Rodentia, known as 'dessert rats' are the main hosts especially in Central Asia (Molyneux and Ashford, 1993).

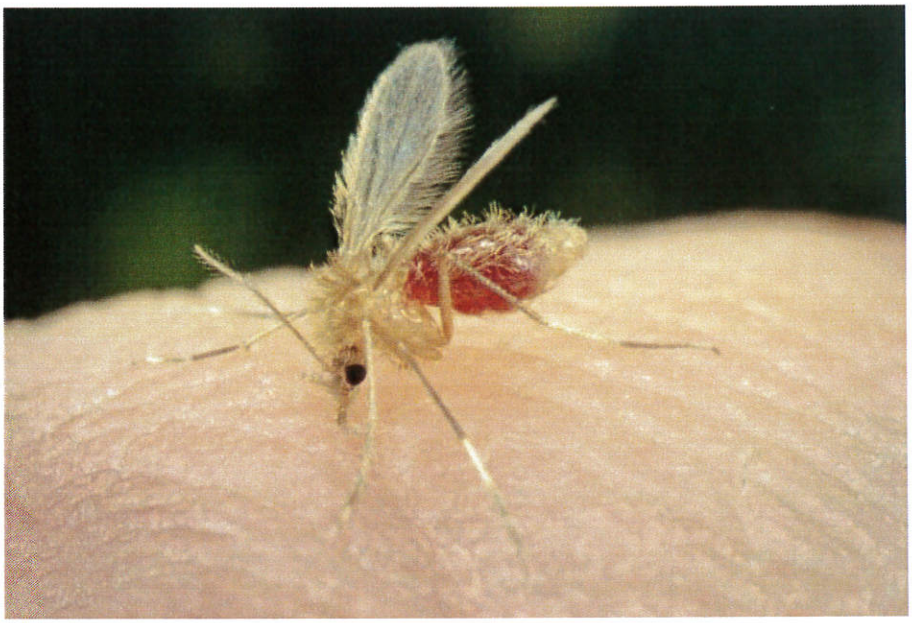

Figure 2.3: *P. papatasi* sandfly landed on human skin surface. Adopted from "*Leishmania major*" (2010).

Despite of the increased infection incidence worldwide, CL still remains as one of the so-called neglected diseases with little interest from financial donors, public health authorities, and professionals to implement activities to research in preventing, controlling or treating the disease. A CL lesion caused by *L. major* usually heals spontaneously but it results in a disfiguring, atrophic scar with pitted appearance, consequently creating a high burden of social stigma (Pace, 2014). Therefore, systemic chemotherapy is important for shortening the disease duration, management of multiple and/or large lesions, especially on the face, improving the cosmetic aspect of scarring, prevent dissemination to the mucosa and avoiding its bad economic impact (Eissa *et al.*, 2011; Blum *et al.*, 2004; Soto and Berman, 2006).

### 2.1.2 Amastigote, promastigote and *Leishmania* spp life cycle

The target for chemotherapy of leishmaniasis is the intracellular amastigote in the mammalian host. The intracellular amastigote stage is the relevant stage used for *in vitro* drug testing as the two stages; extracellular promastigote and intracellular amastigote, are different morphologically, in biochemical and molecular processes. Major biochemical differences such as surface membrane proteins (Beverley and Turco, 1998; Croft and Yardley, 2002) and gene expressions (Charest and Matlashewski, 1994; Croft and Yardley, 2002) explain the difference in drug sensitivity between the two stages of parasites. This later statement is proven by clinical treatment data which showed more than 100 fold sensitivity of intracellular amastigotes than extracellular promastigotes to the standard drugs used for the treatment of leishmaniasis in many cases (Callahan *et al.*, 1997; Ephros *et al.*, 1997; Sereno and Lemesre, 1997; Croft and Yardley, 2002).

*Leishmania* spp infections typically originate via the bite of tiny and-colored blood-feeding flies which in the range of size 3 to 4 mm. These flies, also known as sandflies belonging to either *Phlebotomus* spp (in Europe, North Africa, the middle east and Asia – Old World) or *Lutzomya* spp (from southern USA to northern Argentina – New World) (Beverley and Turco, 1998; Reithinger *et al.*, 2007). In the natural life cycle of *Leishmania* spp, it is transmitted through the bites of infected female sandflies, which is the main vector.

*Leishmania* parasites are dimorphic organisms, which exist in two morphological forms in their life cycle (Figure 2.4). It begins with a non-motile aflegellated amastigote in the midgut of the main vector. The amastigote form develops into a 15-25 μm long by 2-3 μm wide flagellated promastigote once in the insect's gut (Tiuman *et al.*, 2011; Croft and Yardley, 2002). Promastigotes normally appear to have a small fixed body shape and large flagellum. This small shape and large flagellum makes both appear to be vibrating under microscopical oberservation (Molyneux and Ashford, 1983). This will continue for 1 to 2 weeks until they are fully developed before migrating to the vectors' salivary glands and on to the proboscis (Bates, 2007).

When a sandfly takes a blood meal from the host, promastigotes are released into the skin and bloodstream via saliva. These promastigotes are then phygocytosed by, or invade the host macrophage. Within the phagolysosome of resident macrophages, these promastigotes are then transformed into 1 to 3 μm diameter spherical shape amastigotes (Reithinger *et al.*, 2007; Tiuman *et al.*, 2011; Croft and Yardley, 2002). The amastigotes then multiply causing a burden to host cells which are prone to lysis, releasing amastigotes which then may infect other macrophages, thus retaining their survival in the host (Seifert, 2011).

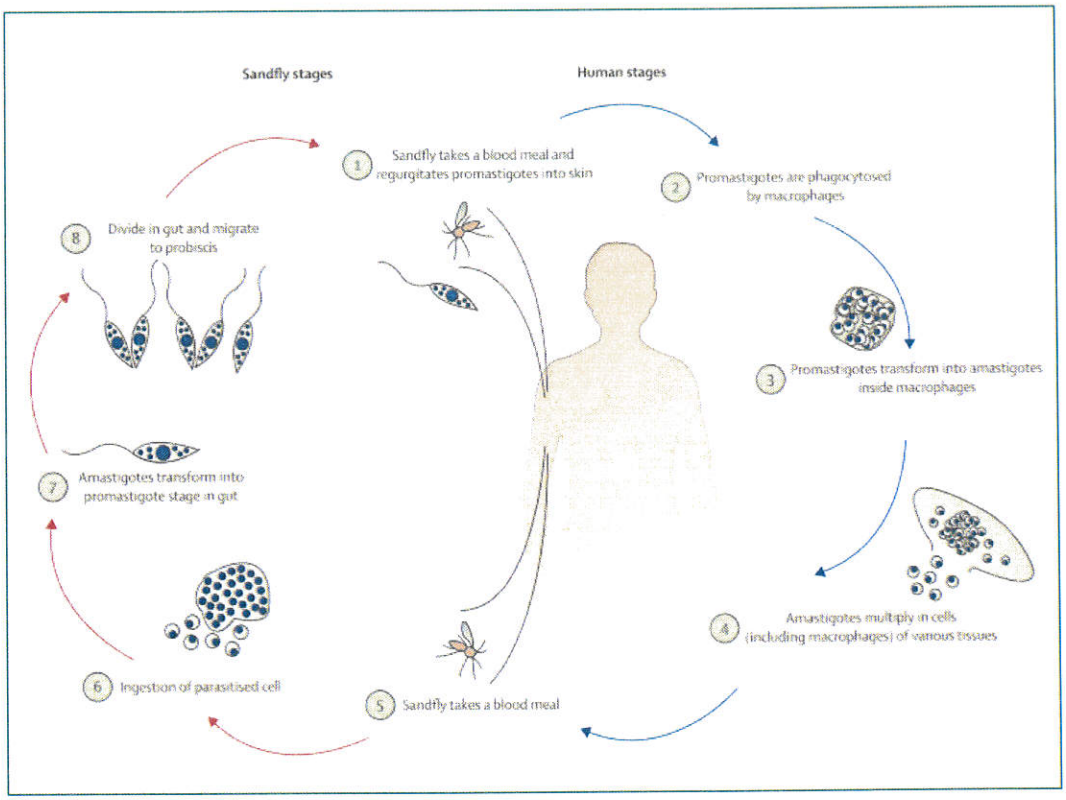


Figure 2.4: Life cycle of the leishmania parasite (Reithinger *et al.*, 2007).

The life cycle of this parasite will be completed when the sandfly blood-feeds on an infected host which housing the amastigotes. When this happens, the infected macrophages of the host are ingested by the sandfly. The macrophages are then digested and the amastigotes that are released transform into promastigotes. Finally, in the insect's gut, promastigotes multiply and give rise to non-dividing metacyclic promastigotes, which are able to establish infection in a mammalian host (Reithinger *et al.*, 2007; Bates, 2007).

### 2.1.3 Symptoms and diagnosis

VL infection is established through the lymphatic and vascular systems with internal organs affected such as bone marrow, liver and spleen (Chan-Bacab and Pena-Rodriguez, 2001). An infected person will experience weight loss, enlarged liver, spleen and lymph nodes, fatigue and fever associated with rigor and chills (Chappuis *et al.*, 2007). On the other hand, CL begins with a papule which develops at the site of a sandfly bite. The papule slowly enlarges and begins to necrotize at the centre (of the papule). Necrosis may be either in the form of 'moist' ulcer characteristics caused by acute *L. major* infection, or the 'dry' ones caused by *L. tropica*. Usually, it takes a few weeks for the infection to appear as a papule, and the ulcers usually appear 2 to 3 months after the sandfly biting season (Molyneux and Ashford, 1983).

Detection of the parasites' DNA in blood or tissue smears has been successful through the development of PCR-based methods, particularly in cases with low parasitic load. However, this method requires a high cost, technical expertise and laboratory infrastructure. Culture methods which involves biopsy aspirates can be used. However, they are time consuming and require culture media and technical expertise. Microscopic

examination of Wright-Giemsa-stained slides from biopsy smears or aspirates is perhaps the most common diagnostic approach used because it is not costly and time consuming. Nevertheless, the technical aspect should be considered during biopsy or aspiration of the affected area, for example, aspiration from bone marrow (Reithinger *et al.*, 2007; Soto and Berman 2006).

### 2.1.4 Current treatment

Leishmaniasis treatment is complicated because of the intramacrophagic location of the infectious form. In a real victim situation, they suffer from immune deficiency and are not able to eliminate the parasites via natural defense mechanism. In addition, certain cases of leishmaniasis are closely associated with malnutrition (Mangesha *et. al.*, 2014). Parallel infections with HIV/AIDS, malaria and pneumonia cause increment in death if untreated in time. The current challenge in treating leishmaniasis is the resistance to drug by parasites species (Carvalho *et al.*, 2000; Rocha *et al.*, 2005; Decuypere *et al.*, 2012).

To date, pentavalent antimony also known as antimonials still acts as the primary therapy as no vaccines exist either for VL, CL or even rarer ones (Croft and Yardley, 2002; Reithinger *et al.*, 2007). Antimonials, such as meglumine antimonate (Glucantime$^{TM}$) and sodium stibogluconate (SbV, or the commercial name is Pentostam$^{TM}$) have been used in the treatment of VL and CL for more than 60 years. The side effect is serious cardiotoxicity which globally affects 9-10% of treated patients. Despite of the 95% cure rates worldwide, SbV has developed acquired resistance in high-prevalence region of India (Seifert, 2011). If this treatment fails, few other drugs can be employed based on the species of leishmania concerned and the available resources.

The most widely use secondary therapy is amphotericin B (Amp-B) which is highly active but causes toxicity to the host cell, alters renal function in approximately 80% of treated individuals (Chan-Bacab and Pena-Rodriguez, 2001) and requires long courses of parenteral administration (Amato *et al.*, 2008). There are different formulations of Amp-B available as it is in crystal form and insoluble in water. Fungizone®, the commercial name for Amp-B deoxycholate which is in unilamellar liposomal formulation, is one of the formulations with high activity against *Leishmania* spp infection (Tiuman *et al.*, 2011). In the case of high rates of unresponsive to antimonials, Fungizone® is used as the first line of treatment. Otherwise, it is the second. However, it can cause few drawbacks including requirement of close monitoring of patients, need for hospitalization, long treatment duration and infusion-related adverse events such as fever, chills and trombophlebitis (Alvar *et al.*, 2006).

AmBisome®, a lipid based or liposomal formulation of Amp-B, has been approved for VL treatment by the U.S. Food and Drug Administration (FDA) (Meyerhoff, 1999). Despite shorter treatment courses and single dose regimes, it may cause a higher relapse rate than Fungizone® (Sundar *et al.*, 2004; Sundar *et al.*, 2003; Sundar *et al.*, 2010). Other formulations of Amp-B such as lipid complex Abelcet® and colloidal dispersion Amphocil® have also been evaluated in clinical trials for VL and/or MCL. AmBisome® (25 mg/kg) is found to successfully reduce the size of lesions caused by *L. major*. However, it is too expensive to use in the majority of endemic countries (Croft and Yardley, 2002).

Miltefosine, an alkylphospholipid, developed as an oral anti-cancer drug and for cutaneous cancers, has subsequently been applied to treat leishmaniasis (Alvar *et al.*, 2006; Sindermann and Engel, 2006; Seifert, 2011). The discovery that miltefosine is

effective against *Leishmania* spp has led to the identification of a modern group of antiprotozoal medicines. In clinical studies, Impavido™, the commercial name for miltefosine was approved and has become the first oral treatment for leishmaniasis in some countries (Croft and Engel, 2006). It is an effective treatment for VL and CL, including for antimony-resistant infections. It also acts as the second line treatment for CL in Columbia and Bolivia (Soto *et al.*, 2001; Alvar *et al.*, 2006). However, this drug may not necessarily be superior in parenteral therapies for all forms of leishmaniasis (Berman *et al.*, 2006). It requires a long course of treatment (28 days) which may make parasites to develop resistance to the drug (Tiuman *et al.*, 2011). Adverse side effects of the drug are observed in the gastrointestinal tract with symptom of hyperplasia of stomach cells and contraindications in pregnancy ladies (FDA, 2013; Sindermann and Engel, 2006).

Paromomycin (Aminosidine®) is an aminoglycoside antibiotic and is currently the latest drug used and has been registered for VL in India (Seifert, 2011). This drug has been used for the treatment of both VL and CL since its identification in the 1960s (Croft *et al.*, 2002; Croft *et al.*, 2006). Poor bioavailability has led to the development of a parenteral formulation for VL and topical formulation for CL. Paramomycin ointment works on CL (Croft *et al.*, 2002) but resistance has been observed in *in vitro* experiment showing that *L. donovani* promastigote has specific and stable resistance towards paramomycin (Maarouf *et al.*, 1998; Seifert, 2011).

Pentamidine, an aromatic diamine, is the isothionate salt also known as Pentacarinat® which still used as the first line of treatment for certain forms of CL that caused by *L. panamensis* and *L. braziliensis* (Seifert, 2011; Croft and Yardley, 2002). However, it is a second line treatment in cases with antimony-resistance, toxicity or

efficacy issues (Alvar *et al.*, 2006). Toxicity, mainly nephrotoxicity is a major safety concern in patients. In addition, there are reports of diabetes cases from VL patients in India, which is causing this treatment to be abandoned (Olliaro *et al.*, 2005).

Other drugs which are still tested in clinical trials are imiquimod, anti-fungal azoles such as ketoconazole, fluconazole, itraconazole, sitamaquine and other new Amp-B formulations. There are also drugs or formulation which are in lead optimization stage and preclinical phase such as licochalcone A which isolated from a Chinese plant named *Glycyrrhiza*, isopropylquinolines which isolated from a plant called *Galipea longiflora* in Bolivia, indolyl quinolines which are quinoline derivatives and buparvaquone derivatives (Seifert, 2011; Croft *et al.*, 2006).

### 2.1.5 Antileishmanial drug discovery assays

There are a few assays being developed by researchers such as *in vitro* assay on promastigotes, *in vitro* assay on macrophage-amastigote models, *in vitro* assay on axenic amastigotes, *in vitro* automated screening assay and *in vivo* assay.

Promastigote assays, also known as leishmanicidal assay or extracellular promastigote assay, are useful cytotoxicity indicators in bioassay guided fractionation of plant products as the activity of the products against this extracellular stage is easy to determine, which is through established viability assay format (Croft *et al.*, 2006). However, the promastigote stage of the parasite is not the infective form in vertebrate hosts. This assay only gives possible leishmanicidal activity of the metabolite or drug tested (Chan-Bacab and Pena-Rodriguez, 2001). This methodology has been adopted by Siqueira-Neto and his colleagues (2010) at the Institute Pasteur Korea (IPK), to be developed as a High-Throughput Drug Screening (HTS) automated system for antileishmania in the identification of active compounds from a large number of

candidates using resazurin (AlamarBlue®) and transfected parasites. This has been successful (Okuno *et al.*, 2003; Singh and Dube, 2004) but not within the clinically relevant amastigote-macrophage model. Various research groups have successfully transfected reporter genes into *Leishmania* spp. However, the majority of them require drug selection to maintain the plasmid (Buckner *et al.*, 1996) which is not ideal for use in drug experiments (Croft *et al.*, 2006). Besides that, verification of results obtained is needed using the macrophage-amastigote or intracellular parasite assay model (Siqueira-Neto *et al.*, 2010).

In *L. major* infections, amastigote-infected macrophages in the dermis of the skin are the targets for drug delivery (Yardley and Croft, 2000). The amastigote-macrophage *in vitro* model, also known as the *in vitro* antileishmanial intracellular amastigote assay, is the most widely used model for testing drugs against *Leishmania* spp. This assay model involves using either murine peritoneal exudate macrophages (PEM) harvested from mouse J774 or human-monocyte THP-1, U937 or HL-60 transformed macrophages as host cells (Croft *et al.*, 2006). Scientists have also suggested that macrophage activation has a significant effect on standard drug accumulation and intracellular parasite killing (Murray, 2001) and mimics the *in vivo* situation as the parasites are rapidly phagocytosed and transformed to amastigotes (Molyneux and Ashford, 1983). In these differentiated non-dividing macrophages, drug activity can be clearly measured or determined by either colorimetric or fluorometric methods (Siqueira-Neto *et al.*, 2010), or microscopically counting the percentage of infected macrophages (Croft *et al.*, 2006; Seifert *et al.*, 2010).