Physicochemical and *In Vitro* Evaluation of Anti-Leishmania Activity of Parvaquone and Related Compounds

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

Signed …… Date ……

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This thesis has been submitted for examination with our approval as university Supervisors

Signed …… Date ……

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Dr Willy Tonui

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DEDICATION

I dedicate this thesis to my loving wife, Louisa for her unwavering support and my parents who tirelessly encouraged me throughout my studies.

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LIST OF ABBREVATIONS

ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity
BCG	Bacilli Calmette-Guerin
BCS	Biopharmaceutical Classification System
CBRD	Centre for Biotechnology Research and Development
CC_{50}	Cytotoxic Concentration
CL	Cutaneous Leishmaniasis
DCL	Diffuse Cutaneous Leishmaniasis
DMFA	Dimethyl Formalaldehyde
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
GIT	Gastro Intestinal Tract
HTS	High Throughput Screening
IR	Infection Rate
KEMRI	Kenya Medical Research Institute
KHP	Potassium Hydrogen Pthalate
Log D	Distribution coefficient
Lop P	Partition coefficient
MIC	Minimum Inhibitory Concentration
NaOH	Sodium Hydroxide

NCE	New Chemical Entity
NIAID	National Institute of Allergy and Infectious Diseases
NNN	Novy-MacNeal-Nicolle media
NQ	2-Hydroxy -1, 4- Napthoquinone
OECD	Organization for Economic Cooperation and Development
P_{app}	$Caco_2$ monolayer Permeability
pKa	Ionization constant
PNQ	2-Hydroxy-3-(2-Phenylbutyl)-1, 4- Naphthoquinone
QSAR	Quantitative Structure Activity Relationship
RPMI	Roswell Park Memorial Institute medium
SOPs	Standard Operating Procedures
THF	Tetrahydrofolate
VL	Visceral Leishmaniasis
WHO	World Health Organization

ABSTRACT

Leishmaniasis is a widespread parasitic disease caused by protozoa of the genus Leishmania. The control of leishmaniasis remains a problem and it is emerging as an important opportunistic infection in immuno-compromised patients especially those infected with HIV. The current treatment use pentavalent antimony as primary therapy, which must be administered parenterally and requires long duration of therapy. It also has toxic side effects and variable efficacy with treatment failures being reported in India and Kenya. The most widely used secondary treatment is amphotericin B which is highly active but has extensive toxicity complications with the newer formulations being too expensive for use by the majority of endemic countries. A real need exists for improved anti-leishmanials as the current chemotherapy is inadequate and expensive. This study evaluated the physicochemical profiles comprising solubility, ionization constant ($pKa_)$ and partition coefficient (Log P) of parvaquone, buparvaquone, 2-Hydroxy-1, 4-Naphthoquinone (NQ) and 2-Hydroxy-3- (2-phenylbutyl)-1, 4-Naphthoquinone (PNQ). Parvaquone and buparvaquone are hydroxynapthoquinones that were developed for treatment of East coast fever (ECF) in cattle and being tested here against leishmaniasis. The anti-leishmanial activity of these compounds was also determined in cell free media and infected macrophages. Aqueous Solubility, pKa and Log P measurements were done following literature methods of poorly soluble drugs. Amphotericin B and Pentostam were used as standard drugs in *in vitro* evaluation of anti-leishmania activity. The compounds under test exhibited low aqueous solubility as all were below 65 µg/ml. According to literature most drugs that are orally active have

aqueous solubility greater than 65 µg/ml but when the solubility is 20 µg/ml or less as was the case here, the probability of useful oral activity is very low. In cell free and macrophage assay, buparvaquone displayed the most potent activity while 2- hydroxy-1, 4-Naphthoquinone (NQ) had the least potency among the test drugs. The *in vitro* activity of buparvaquone was comparable to standard drugs unlike parvaquone that exhibited weak activity compared with the positive control. The results further showed that the mode of action of the test drugs was not through nitric oxide production. In cytotoxicity assays, buparvaquone had the lowest minimum inhibitory concentration (MIC) among test drugs and a high selectivity index (SI). The test compound, 2-hydroxy-3- (2-phenylbutyl)-1, 4- Naphthoquinone (PNQ) had the lowest SI. In conclusion, buparvaquone exhibited the most favourable physico chemical parameters and *in vitro* activity among the test drugs. However, the challenge for oral and topical formulation of this drug is its poor aqueous solubility which will lead to low bioavailability.

CHAPTER 1: INTRODUCTION

1.1 Background

Leishmaniasis is a complex of disease syndromes, with a spectrum that has classically been divided into visceral, cutaneous and mucocutaneous forms caused by protozoal parasites of the genus Leishmania. The disease is endemic in manly tropical and sub-tropical regions of the World with 350 million people at risk (WHO, 1990). There is an estimated prevalence of 12 million (WHO, 1990) with an incidence of 1.0 to 1.5 million cases per annum of the disfiguring cutaneous leismaniasis (CL) and 0.5 million cases per annum of the potentially fatal visceral leishmanasis (VL) (Ashford and Desjeux, 1992). Although visceral leishmaniasis is endemic in 82 countries, over 90% of cases are found in three regions namely East Africa (Kenya, Sudan and Ethiopia), Asia (India, Bangladesh, Nepal) and South America (Brazil) (WHO, 1991; 1996). In the year 2000, the disease burden associated with visceral leishmaniasis measured in disability-adjusted life years was estimated to be two million (WHO, 1996).

Over 20 species of Leishmania have been identified, and the most common species that cause CL in the old World are *L. major*, *L. tropica* and *L. aethiopica* and in the new World *L. braziliensis, L. panamensis, L. amazonensis* and *L. mexicana* (Croft and Yardley, 2002). The Old World refers to Africa, Europe and Asia while the New World refers to South and North America. Typically, CL presents as a nodule at the site of an insect bite, that gradually develops into an open lesion before self-cure within 3-18

months. VL is mainly caused by *L. donovani* (Croft and Yardley , 2002) in which the parasite is spread from the site of inoculation to multiply as an amastigote in macrophages of the spleen, liver, lymph nodes and bone marrow. The onset of VL may be gradual, with months of fever, splenic discomfort, abdominal distension, weight loss, dry cough, edema or diahorhea. In epidemics, the illness is often acute with high fever and rapid progression to prostration, wasting, cough and severe anaemia (Seaman *et al*., 1996; Perea *et al.*, 1991; Zijistra *et al.* 1991; 1992).

1.2 Treatment of Leishmaniasis

Pentavalent antimonials have been the main drugs used for treatment of visceral leishmaniasis since the 1940's. Though these drugs have been effective, their main drawback is that they have to be administered by injection (parenterally). They also have undesirable side effects and variable efficacy against VL and CL (Mantyla *et al.*, 2004). The demand for new anti-leishmanial drugs has acquired prominence in recent years due to demonstration of resistance to pentavalent antimonials, the first line chemotherapy (Croft and Coombs, 1999).

The concept of drug resistance in leishmaniasis is not straight forward. Sensitivity to drugs has to be evaluated carefully and considered in relation to the differences in intrinsic drug sensitivity between species and situations where the disease is an anthroponotic as opposed to Zoonotic. Treatment efficacy is also compromised when there is immunosuppression in particular due to HIV infection. This can lead to exacerbation of disease or emergence from latent infection, the depleted immune

capability means that standard chemotherapy is frequently unsuccessful (Croft and Coombs, 1999).

The second line drugs such as amphotericin B have either toxic side effects or are expensive (Croft and Yardley, 2002). Other drugs that have been used in the treatment of leishmaniasis with limited success include miltefosine, sitamaquine, imiquimod and paramomycin. Though a real need for new anti-leishmanials exist successful development of new drugs is rare because the disease is prevalent in poor countries and therefore unattractive to pharmaceutical companies (Kayser *et al.*, 2003 a).

1.3 Development of new drugs

New anti-leishmanial drugs are required and the favoured approach to achieve this goal is to identify potential drug targets, validating them and then identifying inhibitors that can serve as lead compounds to enter a drug development process (Barret *et al*., 1999). One of the major gaps in drug development process for anti-leishmanials has been the chemistry-biology interaction to obtain lead compounds that can enter the development process. More chemical syntheses are required to obtain analogues of active compounds that can lead to the discovery of new drugs. However, the challenge has been obtaining chemical libraries that can be screened to discover novel inhibitory compounds and ensuring patent protection (Croft and Coombs, 1999).

The development process itself is highly costly. As the resources available for the search for new anti-leishmanials are considerably more restricted than those allocated for disease directly affecting the developed World, few candidate drugs have progressed

into development over the years. The most fruitful recent approach has been piggy-back upon the drug development programmes in other areas, such as anti-cancer and anti-fungal agents. The advent of miltefosine as a registered anti-leishmanial agent is a prime example of the success of this approach (Croft and Coombs, 1999).

In drug development the three main reasons for clinical failure of a new chemical entity (NCE) are lack of efficacy, toxicity and unfavourable pharmacokinetic properties. All three are poorly understood and difficult to predict. An estimated 30% of drug candidate molecules are rejected due to phamacokinetic related failures as a result of poor solubility and poor permeability (Avdeef, 2001). Hence, drug discovery research is under enormous pressure to increase the probability of ultimate success of the drug entering clinical trials (Rodrigues, 1998).

Given these issues, there has been a concerted effort towards better prediction of pharmacokinetic properties and toxicity of NCEs in early discovery efforts. Through progress in linking *in silico* (computer based predictions) and higher throughput physicochemical methods with *in vitro* approaches, progress is being made in predicting pharmacokinetic properties (Smith and Waterbeemd, 1999). Such High Throughput Screening (HTS) efforts require rapid methods and use of minimum amounts of compounds, so that new molecules can be synthesized upon the predicted or experimental outcomes of pharmacokinetic and/or toxicity experiments in a timely fashion (Chaturvedi *et al*., 2001).

Partition coefficient (log P) and ionization constant (pK_a) are useful physicochemical parameters in understanding the behaviour of drug molecules. The absorption, distribution, metabolism and excretion (ADME) profile of a drug is related to its Log P value and therefore in combination with pK_a can be used to predict the distribution of a drug molecule in a biological system (Avdeef, 2001).

CHAPTER 2: LITERATURE REVIEW

2.1 Leishmaniasis the disease

Leishmaniasis is a parasitic disease found in about 88 countries (Singh & Sivakumar, 2004). Approximately 350 million people live in these areas. It is widespread in many tropical and sub-tropical regions of the World where it constitutes a serious health problem (Mello *et al.,* 2004). The foci in which leishmaniasis is found range from rain forests in Central and South America to deserts in West Asia. More than 90% of the global cases of visceral leishmaniasis are in India, Bangladesh, Nepal, Brazil and Africa (Croft *et al.,* 2006). The annual global number of new cases of cutaneous leishmaniasis is estimated at 1.5 million compared to visceral leishmaniasis at 500,000 (Croft *et al.,* 2006). In Southern Sudan, 30,000 - 40,000 people have died from leishmaniasis or related complications. Villages have lost 30 - 65% of their population to visceral leishmaniasis, up to 40% carry the parasites, and 30 - 40% are immune to it (Davidson and Croft, 1992). The epidemic is extending to the North. Other endemic areas in Africa include Kenya, many Western and Central African countries, and all the countries in Northern Africa (Davidson and Croft, 1992). People of all ages are at risk of contracting leishmaniasis if they live or travel where the disease is endemic. It is more common in rural than urban areas, but it is found in the outskirts of some cities (Singh and Sivakumar, 2004).

In Kenya, the disease has been reported in Baringo, Kitui, Machakos, Meru, West Pokot, Elgeyo Marakwet and Turkana districts, where *kala-azar* or visceral leishmaniasis (VL)

is (Fendall, 1961). Outbreaks of VL were reported in the previously non-endemic districts of Wajir and Mandera in North - Eastern Kenya between May 2000 and August 2001 (Marlet *et al.*, 2003). The aetiological agents for CL include *Leishmania major* which has been reported in Baringo, *L. tropica* in Laikipia, Samburu, Isiolo, Nakuru and Nyandarua districts while *L. aethiopica* has been reported in the Mt Elgon area (Tonui, 2006).

2.1. 2 The Leishmania life cycle

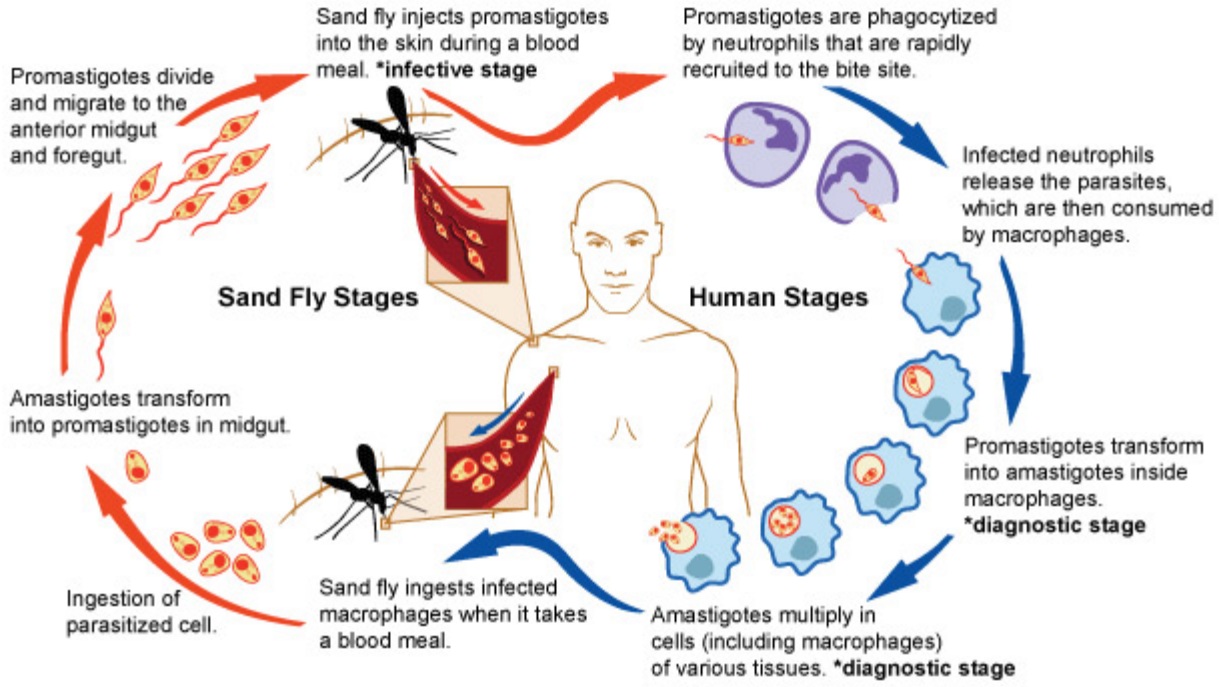
Leishmaniasis is a parasitic disease caused by over 15 different species of the protozoan parasite in the genus Leishmania (Croft *et al.*, 2006). The most common species that cause cutaneous leishmaniasis in the Old World (Europe, Asia and Africa) are *L. major, L. tropica* and *L. aeithiopica*, and in the New World (America) *L. braziliensis, L. panamensis, L. amazonensis* and *L. mexicana* (Kayser *et al.*, 2003).

The only proven vector of the Leishmania parasite is the blood-sucking female of the genus Phlebotomus in the Old World and Lutzomyia in the New World. The sand fly vector becomes infected when feeding on the blood of an infected individual or an animal reservoir host (Fig 1). The Leishmania parasites live in the macrophages as round, non-motile amastigotes (3 - 7 micrometers in diameter). The fly ingests the macrophages during the blood meal and the amastigotes are released into the gut of insect. Almost immediately the amastigotes transform into the motile, elongated (10 - 20 micrometers), flagellate promastigote form. The promastigotes then migrate to the alimentary tract of the fly, where they live extracellularly and multiply by binary fission.

Four to five days after feeding the promastigotes move forward to the oesophagus and the salivary glands of the insect (Croft and Yardley, 2002; Hommel, 1999).

When the sand fly next feeds on a mammalian host, its proboscis pierces the skin and saliva containing anti-coagulant is injected into the wound to prevent the blood from clotting, the Leishmania promastigotes are transferred to the host along with the saliva (Fig 1). Once in the host the promastigotes are taken up by the macrophages where they rapidly revert to the amastigote form. The Leishmania are able to resist the microbiocidal action of the acid hydrolases released from the lysozymes and so survive and multiply inside the macrophages, eventually leading to the lysis of the macrophages. The released amastigotes are taken up by additional macrophages and so the cycle continues (Hommel, 1999).

The ability of Leishmania parasites to establish an infection in humans is dependent upon the adaptation of parasites to survive and multiply in macrophages and on the consequent host immunosuppression. Cure or limitation of the infection is dependent upon an effective immune response that activates macrophages to produce requisite toxic nitrogen and oxygen metabolites that kill intracellular amastigotes (Alexander *et al.,* 1999). One rational approach to therapy is to modulate immune response to overcome the negative control systems and to boost the killing responses.



Source: NIAID.

Figure 1. Diagram showing the sand fly and human stages of leishmaniasis

2.1. 3 Forms of leishmaniasis

There are different forms of leishmaniasis. The disease ranges from self-healing ulcers (cutaneous leishmaniasis, CL) to progressive nasopharyngal infections (mucocutaneous leishmaniasis, MCL) to debilitating visceral leishmaniasis (VL). While CL poses essentially cosmetic problems and MCL leads to painful disfiguration, social stigmatization and often highly severe secondary infections. The most common forms are CL, which causes skin sores, and VL, which affects some of the internal organs of the body (spleen, liver, bone marrow). Victims of CL have one or more sores on their skin. The sores can change in size and appearance over time. They often end up looking

somewhat like a volcano, with a raised edge and central crater. Some sores are covered by a scab. The sores can be painless or painful. Some leishmaniasis patients may have swollen glands near the sores like under the arm if the sores are on the arm or hand (Fournet *et al.*, 1996).

VL is mainly caused by *L. donovani* in which the parasite is spread from the site of inoculation to multiply as an amastigote in macrophages of the spleen, liver, lymph nodes and bone narrow (Singh, 2006; Croft and Yardley, 2002). Victims of VL usually have fever, weight loss, and enlarged spleens and livers. Leishmaniasis patients usually have low blood counts, including a low red blood cell count (anemia), low white blood cell count, and low platelet count (Bhattacharya *et al.*, 2006). VL is generally lethal if left untreated (Mantyla *et al.*, 2004; Kayser *et al.*, 2003 a).

2. 2 Control of leishmaniasis

2.2.1 Leishmaniasis vector control

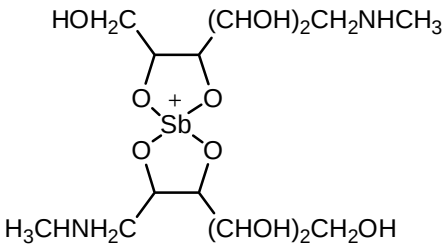
Spraying houses with insecticide is the most widely used for controlling sandflies that are endophilic (rest mostly indoors after feeding). House spraying with the pyrethroid *lambda-cyhalothrin* reduced the odds of CL in Kabul by 60% and reduced the risk of CL in the Peruvian Andes by 54% (Davies *et al.*, 2000; Reyburn *et al.*, 2000). In addition to DDT, malathion, fenitrothion and propoxur, synthetic pyrethroids such as *delta-methrin* have been successfully used for the control of sandflies (WHO, 1990). Where sandflies are endophagic (mainly feed indoors) and most active when people are asleep, bed nets provide considerable protection. Protection is enhanced by treating wide mesh nets with

pyrethroids thus reducing sandfly biting rates by 64 - 100% (Tayeh *et al.,* 1997; Ashford *et al*., 1998; Bern *et al.,* 2000).

2.2.2 Chemotherapy of Leishmaniasis

2.2.2.1 Antimonials

Since the 1940's pentavalent antimony compounds (**1** and **2**) have been the main drugs used in the treatment of VL and some forms of CL (WHO, 1996). The lengthy dosing regimen of these drugs often causes side effects such as myalgia, pancreatitis, cardiac arrhythmia and hepatitis leading to the reduction or ceasation of treatment. The cost of medication varies from US$ 15 -150 per patient while the total cost including hospital stay is estimated at US $ 629 (Guerrin *et al.,* 2002).



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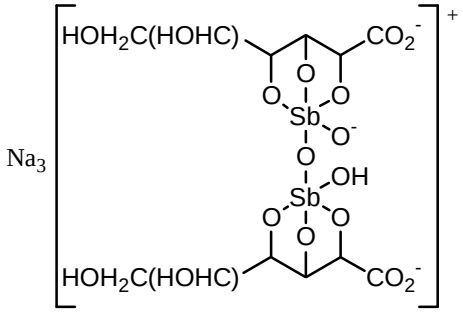

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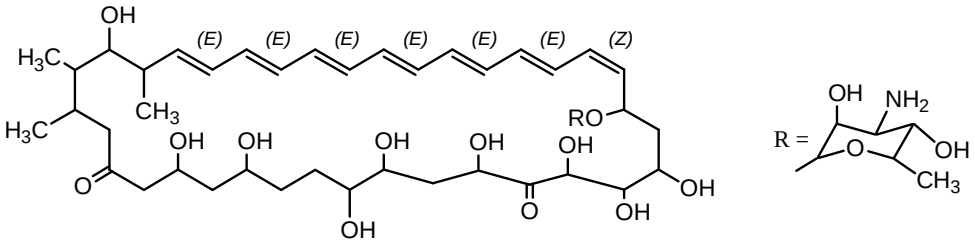
Solutions of Glucantime**, 1** and Pentostam, **2,** remain first line treatment for VL and some forms of CL (Olliaro and Bryceson, 1993; WHO, 1996b; Berman, 1997).

2.2.2.2 Amphotericin B

Amphotericin B **, 3 ,** a macrolide polyene anti-biotic isolated from *Streptomyces nodosus* was first shown to have anti-leishmania activity in the early 1960's and was soon used in the treatment of mucocutaneous Leishmania (Croft and Yardley, 2002). Amphotericin B (amp. B) complexes with 24-substituted sterols such as ergosterol in cell membrane, thus causing pores which alter ion balance and result in cell death (Roberts *et al.*, 2003). Although amp B has long been considered an alternative treatment for mucocutaneous and visceral leishmaniasis (WHO, 1996) its use has been restricted by infusion related and delayed toxic side affects particularly cardiotoxicity and nephrotoxicity (Khoo *et al.*, 1994).



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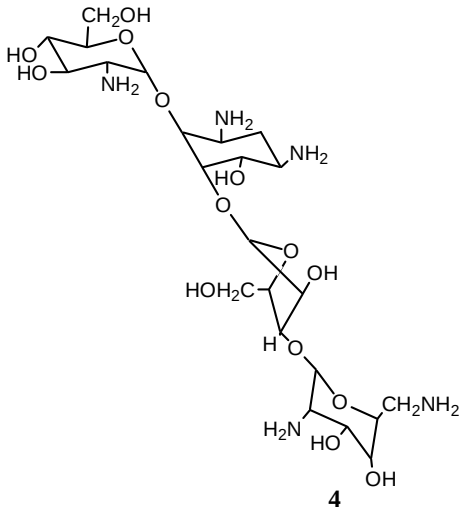
The past decade has seen the development of a number of lipid formulations of amp. B that have greatly reduced toxicity in comparison to the parent drug, for treatment of systemic mycoses and are commercially available. The major limitation that prevents more widespread use of this commercial amp. B lipid formulation is their high cost and

remains beyond the budgets of most endemic countries (Bennett, 2000). The cost of amphotericin B medication is estimated at US$ 60 - 150 per patient while the lipid formulations cost US$ 1000 - 2500 per patient (Guerin *et al.,* 2002).

2.2.2.3 Paromomycin

Paromomycin, **4,** is among a group of aminocyclitol-aminoglycoside anti-biotics, which were originally developed as anti-bacterial agents and have significant anti-leishmanial activity (Pamba *et al.* 1990). In bacteria, paramomycin inhibits protein synthesis by binding to 30S sub-unit ribosomes, causing misreading and premature termination of MRNA translation. In Leishmania, paromomycin also affects mitochondrion (El-On et *al*., 1992).

In the 1980's paromomycin topical formulations were developed for CL and parental formulations for VL (Croft and Yardley, 2002). It has been found that 15% paromomycin in soft paraffin plus 12% quaternary ammonium compound are completely effective whereas other drugs in similar formulations had little or no activity. Although the topical approach is attractive, leishmania amastigotes lie deep in the dermal layer and achieving therapeutic concentrations is difficult (Croft and Yardley, 2002).



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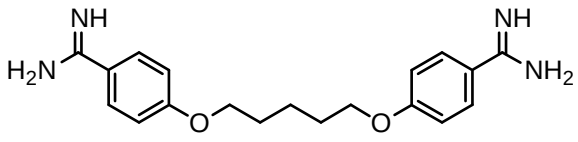
2.2. 2.4 Pentamidine

Pentamidine, **5**, an aromatic diamidine has been used as an alternative treatment for both VL and CL since 1952 and as a primary treatment for *L. aethiopica* diffuse cutaneous Leishmaniasis (DCL). As a second line drug for antimony resistant cases it has proved useful in India and Kenya (Croft and Yardley, 2002). Toxicity has always been a limitation on use with reports of hypoglycaemia, diabetes, nephrotoxicity, tachycardia and pain at the site of injection. There have been attempts to reduce toxicity, for example a liposomal formulation was tested in experimental VL (Banerjee *et al*. 1996).



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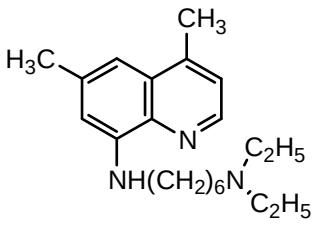
2.2.2.5 Sitamaquine (WR6026) and 8- aminoquinolines

There has been sporadic interest in the anti-leishmanial activity of 8-aminoquinolines since a series of 6-methoxy-8-alkylpiperazinoalkyl aminoquinoline derivatives were shown to have higher activity than pentavalent antimonials and oral availability against *L. donovani* in the hamsters (Beveridge *et al.*, 1958). In subsequent studies, a series of 4-methyl-6-methoxy-8-aminoquinolines (lepidines) were shown to be several hundred times more active than pentavalent antimonials in hamsters (Kinnamon *et al.*, 1978). Structure-activity relationships of methoxy- and hydroxy-substituted compounds were further investigated in a *L. tropica* macrophage model *in vitro* (Berman and Lee, 1983). A summary of the anti-leishmanial activity of 8-aminoquinolines, including the high activity of primaquine in rodent models, is given by Neal (1987).

Sitamaquine, (8-{[6-(diethylamino)hexyl]amino}-6-methoxy-4-methyl quinoline)**,6**, was first synthesized in 1944 and has reached clinical trials. The compound is 708 times more active than meglumine antimoniate against *L. donovani* in hamsters (Kinnamon *et al.*, 1978) and has been on clinical trial for VL in Kenya and Brazil. It is being developed by GlaxoSmithKline and is on phase 2 clinical trials for VL in Kenya (Croft & Yardley, 2002).



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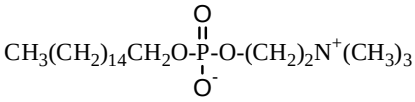
2. 2. 6 Miltefosine and alkyllysophospholipids

Lysophosphatidylcholine analogues were initially studied for their effects on humoral and cellular immunity and only subsequently on tumor growth *in vitro* and *in vivo* (Runge *et al.*, 1980). The studies led to the synthesis of several types of alkyllysophospholipids (ALPs) such as miltefosine **(7)**, edelfosine **(8)**, ilmofosine **(9)** and SRI 62-834 **(10)** that showed anti-cancer activity (Berdel, 1991; Brachwitz and Vollgraf, 1995). The anti-leishmanial activities of this group of compounds were first reported in 1987, when the activity of alkylglycerophosphocholines against *L. donovani* promastigotes was described *in vitro* macrophages and BALB/c mice models (Achterberg and Gercken, 1987; Croft *et al.*, 1987).



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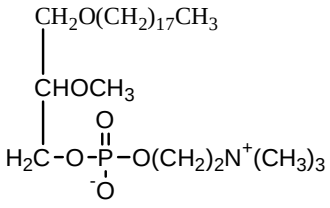

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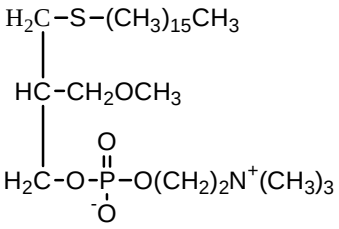

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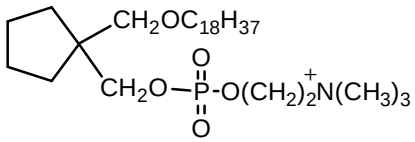

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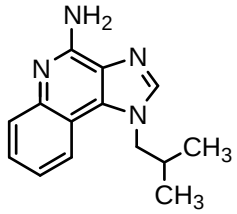
In India, Miltefosine, **7,** had a 95% cure rate when used at a dose of 100 mg/kg for 28 days (Jha *et al.*, 1999; Sundar *et al*., 1999). The drug also proved to be effective against antimonial resistance cases (Croft *et al.*, 1987; Sundar *et al*, 1999). Problems include reversible toxic effects and in treatment of women due to teratogenic properties of the compound.

2.2. 2. 7 Immunotherapy

There have been many studies using endogenous biologicals, microbial derivatives or synthetic compounds in both animals and humans most frequently using immunomodulators that have direct effect on macrophages (Convit *et al.*, 1987; Cillari *et al*., 1994; Ghose *et al*., 1999). A schiff-base forming compound tucaresol that enhances Th1 responses and the production of IL-2 and IFN-γ in mice and humans has activity against infection caused by *L. donovani* in BALB/c and C57B1/6 mice. Imiquimod**, 11** , an imidazoquinoline, used in the treatment of genital warts has a number of immunomodulatory activities and induces release of TNF-ά and IL-1. This drug has been shown to induce nitric oxide production in macrophages *in vitro*, killing

intracellular *L. donovani* amastigotes. A 5% imiquimod cream had a significant suppressive effect on the development of *L. major* infection in BALB/c mice (Buates and Matlashewski, 1999).



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2.2.2.8 Leishmaniasis vaccine development

Of all the parasitic diseases, leishmaniasis is considered the most likely to succumb to vaccination. The parasite has a particularly simple life cycle. First generation vaccines consist of killed Leishmania organisms mixed with a low concentration of bacille Calmette-Guérin (BCG, the vaccine against tuberculosis) as adjuvant. These are currently undergoing field trials in Venezuela for efficacy (Reed, 2004).

Resolution of primary CL usually results in resistance to re-infection, and studies in experimental models have suggested simple CD4 Th1-type, cell mediated resistance, involving activation of macrophage killing mechanisms by T-lymphocytederived γ-interferon. In experimental models of cutaneous leishmaniasis, in which CD4 Th1 responses are driven towards a polarised Th1 response, protection can indeed be achieved by vaccination, although this rarely results in complete protection from

development of lesions. However, such vaccines stimulate poor memory, and protection wanes after a few weeks (Handman, 2001).

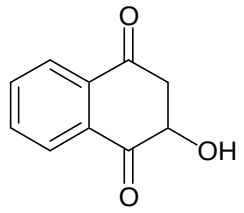
In the past 3 - 4 years the view of the requirements for vaccine induced immunity has changed. A major paradigm shift reflects the role of CD8 T cells. New models of CL indicate that CD8 cells are vital for primary resistance (Belkaid *et al.,* 2002). It has also been recognized that CD8 cells are required for the maintenance of long term vaccine induced immunity. Although the capacity to induce CD8 cell responses is a feature of DNA vaccines, it has also been shown for some protein-based vaccines (Rhee *et al.,* 2002). Although there are concerns about whether the same vaccine will work for all leishmaniasis, for VL, the situation remains less promising. Human trials of vaccines against VL are only likely to follow from successful outcomes from CL, or as in the Sudan, on a compassionate basis (Khalil *et al.,* 2000). Recent reports suggest some progress is being made in vaccines for canine VL (Gradoni, 2001).

Second generation vaccines, which consist of genetically reconstructed *Leishmania* parasites incapable of producing disease, recombinant molecules or their corresponding DNA mixed together in a cocktail vaccine, or recombinant organisms carrying leishmanial genes, are currently undergoing pre-development (Reed, 2004) .

2.2.2.9 Natural products with anti- leishmanial activity

Naphthoquinones are ubiquitously distributed in nature and have been used for centuries in home remedies as well as in cosmetics. Henna is an important example, which is a

paste, prepared from powdered leaves of the plant, *Lawsonia alba,* containing lawsone, **12**, (2-hydroxy-1, 4-naphthoquinone), used for coloring skin and hair (Babich *et al*., 1993). Some naphthoquinone drugs, such as menadione, **13,** (2-methyl-1, 4-naphthoquinone), plumbagin, **14**, (2-methyl-5-hydroxy-1, 4-naphthoquinone), and lapachol, **15**, (2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone) having trypanocidal activities upon different trypanosomes and Leishmania responsible for several human diseases that includes African sleeping sickness (*Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*) Kala-azar (*Leishmania donovani*) and Chagas disease (*Trypanosoma cruzi*) (Salmon-chemin *et al*., 2001). The 1, 4-Naphthoquinone derivatives also elicit several interesting and varied biological responses such as anti - bacterial and anti - fungal (Huang *et al*., 2002 and Tandon *et al.,* 2004). The biological activity of 1, 4-naphthoquinone is mainly due to the presence of two carbonyl groups that have the ability to accept one and/or two electrons to form the corresponding radical anion or dianion species as well as their acid–base properties (Rahimipour *et al*., 1996).



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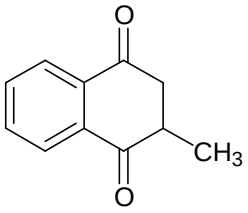

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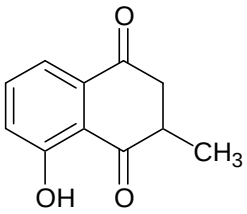

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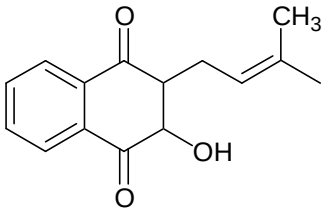

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A study of Tugen, a Kenyan ethnic community in Baringo District, indigenous medicine shows that the most commonly used plant in the treatment of visceral leishmaniasis is *Ajuga remota (ngwadere).* In its preparation, the whole shrub is soaked overnight in water and the filtrate given to the patient to drink. Compounds isolated from this plant that have shown anti-leishmanial activity include; iridoid glucosides, limonoids and terpenoids (Tonui, 2006). Other plants which are used, although to a lesser extent, are *Ziziphus mucronata* (*Tilomwo*) and *Myrsine africana* (*Seketet*). The widespread reliance on *A. remota* is due to its ability to provide relief, the uncertain health delivery system and high cost of western medicine (Kaendi, 1994). Various medicinal plants have been tested for anti-leishmanial activity and hence bioactive compounds isolated (Kayser *et al.*, 2003b).

2.3 Drug Discovery and Development

2.3.1 Evolution of drug discovery and development

The traditional drug discovery process involved initial lead generation on the basis of natural ligands, existing drugs, and literature leads. New compounds would be synthesized and tested for biological activity, and structure–activity relationships would be established for optimization of leads using traditional medicinal chemistry techniques (Smith and O'donnell, 2006). Promising compounds would then be promoted for preclinical and clinical testing and therefore, passed along to the product development staff. While often there was little collaboration between research and development, a few organizations had recognized the importance of discovery-development teams to assess development issues related to new drug candidates (Kaplan, 1972)

The current drug discovery process typically goes through the stages of;

(i) *Target identification* – in which the mechanisms responsible for a disease state are investigated in detail and one or several proteins are identified and validated as suitable targets,

(ii) *Hit identification* – in which large company libraries or smaller focused libraries are screened to find compounds that exert pharmacological activity towards the selected target,

(iii) *Hit-to-lead* – in which the hits are explored with regards to potency, selectivity and absorption, distribution, metabolism, excretion and toxicity (ADMET) properties with the intention being to prioritise and select a few lead series,

(iv) *Lead optimization* – in which pharmacological activity, physicochemical and ADMET properties of lead compounds are optimized to produce up to a few candidate drugs that can enter into the development phase. Computational (Kaplan, 1972) and experimental (Yalkosky and Rubino,1985) methods are used in parallel throughout all of the Phases (i) to (iv) with an emphasis being placed on computational methods in the earlier stages and experimental in the later stages.

Uses of combinatorial chemistry and High Throughput Screening (HTS) technologies have resulted in the generation and selection of increasingly lipophilic drug molecules with potential biopharmaceutical hurdles in downstream development (Lipinski *et al*., 1997). Particularly, the use of organic solvents such as dimethylsulfoxide (DMSO) has contributed to the increase in water-insoluble drugs. Analysis of compound attrition in pharmaceutical development indicated that poor pharmacokinetic factors, i.e., absorption, elimination, distribution, and metabolism (ADME) contributed to about 40% of failed candidates, and for those that moved forward, the development timelines significantly slowed down (Prentiss *et al.*, 1988). To reduce attrition of compounds later in development, pharmaceutical companies began to conduct pharmaceutical, pharmacokinetic, and safety profiling of late- as well as early-phase discovery compounds (Kerns and Li, 2003).

2.3.2 Screening for drugability or developability

Compounds with acceptable pharmaceutical properties, in addition to acceptable biological activity and safety profile, are considered “drug-like” or developable. Typical

acceptable pharmaceutical properties for oral delivery of a drug-like molecule include sufficient aqueous solubility, permeability across biological membranes, satisfactory stability to metabolic enzymes, resistance to degradation in the gastrointestinal tract (pH and enzymatic stability), and adequate chemical stability for successful formulation into a stable dosage form. A number of additional barriers, such as efflux transporters (i.e., export of drug from blood back to the gut) and first-pass metabolism by intestinal or liver cells, have been identified that may limit oral absorption (Ho *et al.*, 2000). A number of computational and experimental methods are emerging for testing (or profiling) drug discovery compounds for acceptable pharmaceutical properties.

2.3.3 Computational tools

Medicinal chemists have always been adept in recognizing trends in physicochemical properties of molecules and relating them to molecular structure. With rapid increase in the number of hits and leads, computational tools have been proposed to calculate molecular properties that may predict potential absorption hurdles. For example, Lipinski's "Rule of 5" (Lipinski *et al*., 1997) states that poor absorption or permeation is likely when:

i) There are more than five H-bond donors (expressed as the sum of –NH and –OH groups).

ii) The molecular weight is more than 500.

iii) Log *P 5* (or *c* log *P* 4.5).

iv) There are more than ten H-bond acceptors (expressed as the sum of Ns and Os)

If a compound violates more than two of the four criteria, it is likely to encounter oral absorption issues. Compounds that are substrates for biological transporters and peptidomimetics are exempt from these rules. The Rule of 5 is a very useful computational tool for highlighting compounds with potential oral absorption issues. A number of additional reports on pharmaceutical profiling and developability of discovery compounds have been published since the report of Rule of 5 (Clark, 2002). Polar surface area (PSA) and number of rotatable bonds have also been suggested as means to predict oral bioavailability. PSA is defined as the sum of surfaces of polar atoms in a molecule. A rotatable bond is defined as any single bond, not in a ring, bound to a nonterminal heavy (i.e., non-hydrogen) atom. Amide bonds are excluded from the count. It has been reported that molecules with the following characteristics will have acceptable oral bioavailability (Veber *et al.,* 2002):

i) Ten or fewer rotatable bonds.

ii) Polar surface area equal to or less than 140 Å2 (or 12 or fewer H-bond donors and acceptors).

2. 3. 4 High-throughput screening (HTS) methods

High-throughput drug-like property profiling is increasingly used during lead identification and candidate selection. HTS pharmaceutical profiling may include: Compound purity or integrity testing, Lipophilicity (log *P*), Dissociation constant (p*K*a), Permeability, Solution/solid-state stability determination. Compound purity (or integrity testing) is important to ensure purity in the early stages because erroneous activity or

toxicity results may be obtained by impure compounds. It is initiated during hit identification and continued into lead and candidate selection.

Solubility is measured to varying degrees of accuracy by HTS methods. Typical methods in the lead identification stage include determination of "kinetic solubility" by precipitation of a drug solution in dimethylsulfoxide into the test medium. Since the solid-state form of the precipitate (crystalline or amorphous) is often not clearly known by this method, the measured solubility is approximate and generally higher than the true (equilibrium) solubility. Kinetic solubility, however, serves the purpose of identifying solubility limitations in activity or *in vitro* toxicity assays or in identifying highly insoluble compounds. It was observed that compounds with a kinetic solubility greater than 65 g/mL (in pH 7 non-chloride containing phosphate buffer at room temperature), poor oral absorption is usually due to factors unrelated to solubility (Lipinski *et. al.*,, 1997).

Estimation or measurement of p*K*a is important to understand the state of ionization of the drug under physiological conditions and to evaluate salt-forming ability (Serajuddin and Pudipeddi, 2002). Log *P* determines the partitioning of a drug between an aqueous phase and a lipid phase. Log *P* and acid p*K*a can be theoretically estimated with reasonable accuracy (Lipinski *et. al.*, 1997; Hilal *et. al*., 1996 and Albert and Serjeant , 1984). High-throughput methods are also available for measurement of log *P* (Donovan and Pescatore, 2002) and p*K*a (Cormer and Box, 2003).

Physical flux of a drug molecule across a biological membrane depends on the product of concentration (which is limited by solubility) and permeability. High-throughput artificial membrane permeability (also called Parallel Artificial Membrane Permeability Assay) has been used in early discovery to estimate compound permeability (Kansy *et. al*., 1998).

This method measures the flux of a compound in solution across an artificial lipid bilayer deposited on a microfilter. Artificial membrane permeability is a measure of the actual flux (rate) across an artificial membrane whereas log *P* or log *D* represent equilibrium distribution between an aqueous and a lipid phase. Sometimes the term "intrinsic permeability" is used to specify the permeability of the unionized form. Artificial membrane permeability can be determined as a function of pH. The fluxes across the artificial membrane in the absence of active transport have been reported to relate to human absorption through a hyperbolic curve. The correlation of permeability through artificial membranes may depend on the specific experimental conditions such as the preparation of the membranes and pH. Therefore, guidelines on what is considered acceptable or unacceptable permeability must be based on the individual assay conditions. For example, Hwang *et. al*. (2003) ranked compound permeation on the basis of the percent transport across the lipid bilayer in 2 h: 2% (low), 2 to 5% (medium), and 5% (high), respectively. Caco-2 monolayer, a model for human intestinal permeability, is commonly used in drug discovery to screen discovery compounds (Artursson *et al.*, 2001; Stoner *et al.*, 2004). The method involves measurement of flux of the compound dissolved in a physiological buffer through a monolayer of human

colonic cells deposited on a filter. Caco-2 monolayer permeability has gained considerable acceptance to assess human absorption. Compounds with a Caco-2 monolayer permeability (P_{app}) similar to or greater than that of propranolol (~30 $x10^6$ cm/sec) are considered highly permeable, while compounds with P_{app} similar to or lower than that of ranitidine ($1x10^6$ cm/sec) are considered poorly permeable. Hurdles associated with determination of permeability of poorly soluble compounds using Caco-2 method have been reviewed (Ingels and Augustijns, 2003).

2.3.4 Biopharmaceutical considerations in dosage-form design

For systemic activity of a drug molecule, it must be absorbed and reach the bloodstream or the site of action if given by the oral, topical, nasal, inhalation, or other route of administration where a barrier between the site of administration and the site of action exists. Even when a drug is injected intravenously or intramuscularly, one must ensure that it is not precipitated at the site of administration and that it reaches the site of action. In the development of dosage forms for a particular drug, a formulator must, therefore, carefully consider various physico-chemical, biopharmaceutical, and physiological factors that may influence absorption and transport of drugs. Appropriate formulation strategies must be undertaken to overcome the negative influences of any of these factors on the performance of dosage forms (Smith and O' donell, 2006).

A large majority of pharmaceutical dosage forms are administered orally, and in recent years, the drug solubility has become the most difficult challenge in the development of oral dosage forms. For example, in the 1970s and 1980s, when dissolution,

bioavailability, and bioequivalence of drugs came under intense scrutiny and many of the related Food and Drug Administration (FDA) guidelines were issued . A drug with solubility less than 20 g/ml was practically unheard of. Presently, new drug candidates with intrinsic solubility less than 1 g/ml are very common (Smith and O' donell, 2006). Aqueous solubility is probably the single most important biopharmaceutical property pharmaceutical scientists are concerned with. It has been the subject of computational prediction for several years (Yalkowsky and Valvani, 1980; Klopman *et al.*, 1992; Huuskonen *et al.*, 1998 and Chen *et al*., 2002). The overall accuracy of the predicted values can be expected to be in the vicinity of 0.5 to 1.0 log units (a factor of 3 to 10) at best. Although a decision on acceptance or rejection of a particular compound cannot be made only on the basis of predicted parameters, these predictions may be helpful to direct chemical libraries with improved drug-like properties (Mckenna *et al*., 2002).

In addition to solubility, physicochemical factors influencing oral absorption of drugs include dissolution rate, crystal form, particle size, surface area, ionization constant and partition coefficient. Among the physiological factors, drug permeability through the gastro intestinal tract (GIT) membrane is of critical importance. Other physiological factors playing important roles in the performance of an oral dosage form are transit times in different regions of the GIT tract, GIT pH profile, and the presence of bile salts and other surfactants. Physiological differences such as the unfed vs. the fed state also need to be considered (Smith and O'donell, 2006).

Since solubility and permeability are the two most important factors influencing oral absorption of drugs, the following biopharmaceutical classification system (BCS) for drug substances, based on the work by (Amidon *et al.*, 1995) has been recommended by the Food and Drug Administration (FDA, 1997):

Class I — Drug is highly soluble and highly permeable

Class II — Drug is poorly soluble, but highly permeable

Class III — Drug is highly soluble, but poorly permeable

Class IV — Drug is both poorly soluble and poorly permeable

For a BCS Class I compound, there are no rate-limiting steps in drug absorption, except gastric emptying, and, therefore, no special drug-delivery consideration may be necessary to make the compound bio-available. On the other hand, for a BCS Class II compound, appropriate formulation strategy is necessary to overcome the effect of low solubility. For a BCS Class III compound, formulation steps may be taken to enhance drug permeability through the GIT membrane, although the options could be very limited. Often, the continued development of a BCS Class III compound depends on whether its low bio-availability from a dosage form because of poor permeability is clinically acceptable or not. A BCS Class IV compound presents the most challenging problem for oral delivery.

Here, the formulation strategy is often related to enhancing the dissolution rate to deliver maximum drug concentration to the absorption site. As for a Class III compound, formulation options to enhance drug permeability are often limited. Because of the

importance of BCS in the development of dosage-form design strategy, an early classification of new drug candidates is essential to identify their formulation hurdles.

2.3.5 Physiological factors affecting oral absorption

2.3.5.1 Membrane physiology

In 1900, Overton performed some simple but classic experiments related to cell membrane structure. By measuring the permeability of various types of compounds across the membranes of a frog muscle, he found that lipid molecules could readily cross this membrane. Larger lipid insoluble molecules couldn't and small polar compounds could slowly cross the membrane. He suggested that membranes were similar to lipids and that certain molecules (lipids) moved across membranes by dissolving in the membrane (Kleinzeller, 1997). These results suggest that the biologic membrane is mainly lipid in nature but contains small aqueous channels or pores (Fig. 2)

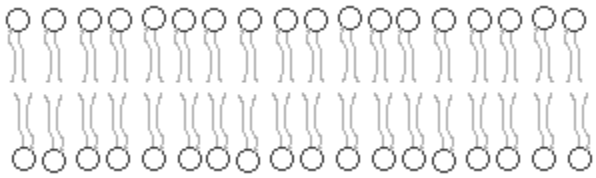


Figure 2. Lipid bilayer

Other experiments involving surface tension measurements have suggested that there is also a layer of protein on the membrane. These results and others have been incorporated into a general model for the biological membrane. This is the Davson-Danielli model (Danielli and Davson, 1935) as depicted in Fig. 3 below .

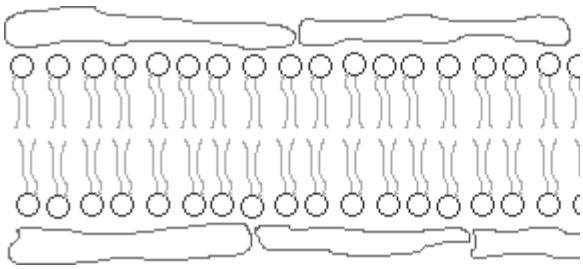


Figure 3 . The Davson-Danielli model

Later work (Danielli, 1975) suggested the presence of "active patches" and protein lining to pores in the membrane (Fig. 4).

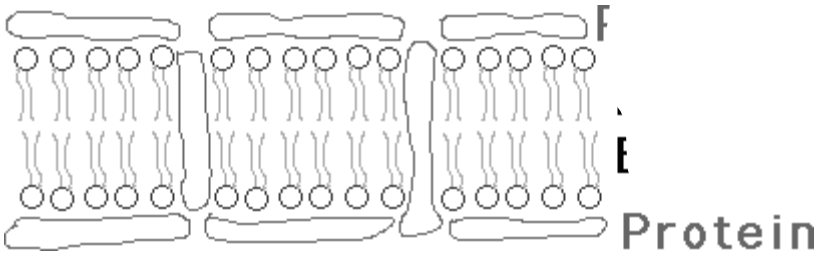


Figure 4. Modified Davson-Danielli model

Work during the 1970s and 1980s suggested the model proposed by Singer and Nicolson 1972 called the fluid mosaic model (Fig.5). With this model the lipid bilayer is retained but the protein drifts between the lipid rather than forming another layer on either side of the lipid bilayer (Singer and Nicolson, 1972).

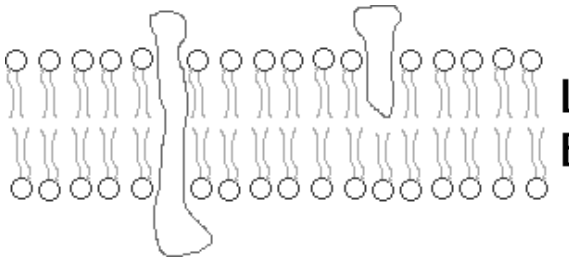


Figure 5. The Fluid mosaic model

The membrane then acts as a lipid barrier with protein formed pores. The protein within the membrane can act as transport enhancers in either direction depending on the protein. The barriers between various organs, tissues and fluids areas will consist of cells of different structure and membranes characteristics. In some cases the cells are loosely attached with extracellular fluid freely moving between the cells. Drugs and other compounds, lipid or not, may freely move across this barrier. In other cases there may be tight junctions between the cells which will prevent non lipid movement (Fig.6).

Figure 6. Cell barrier with tight junctions

2.3.5.2 Passage of drugs across membranes

Most drugs cross biological membranes by passive diffusion. Diffusion occurs when the drug concentration on one side of the membrane is higher than that on the other side.

Drug diffuses across the membrane in an attempt to equalize the drug concentration on both sides of the membrane. If the drug partitions into the lipid membrane a concentration gradient can be established. The rate of transport of drug across the membrane can be described by Fick's first law of diffusion:-

Rate of diffusion = dm / dt = - DA (Ch- Cl) / X …….. (1)

The parameters of this equation are:-

D: diffusion coefficient. This parameter is related to the size and lipid solubility of the drug and the viscosity of the diffusion medium, the membrane. As lipid solubility increases or molecular size decreases then **D** increases and thus dM/dt also increases.

A: surface area. As the surface area increases the rate of diffusion also increase. The surface of the intestinal lining (with villae and microvillae) is much larger than the stomach. This is one reason absorption is generally faster from the intestine compared with absorption from the stomach.

X: membrane thickness. The smaller the membrane thickness the quicker the diffusion process. As one example, the membrane in the lung is quite thin thus inhalation absorption can be quite rapid.

(Ch -Cl): concentration difference. Since V, the apparent volume of distribution, is at least four liters and often much higher the drug concentration in blood or

plasma will be quite low compared with the concentration in the GI tract. It is this concentration gradient which allows the rapid complete absorption of many drug substances.

2.3.5.3 The gastro-intestinal physiology

In any formulation development, the Gastro Intestinal Tract (GIT) physiology that can influence dosage-form performance must be kept in mind. The GIT contents play important roles, since even in the fasted state in humans, the *in vivo* dissolution medium is a complex and highly variable milieu consisting of various bile salts, electrolytes, proteins, cholesterol, and other lipids. The GIT pH is another factor that plays a critical role in the performance of the dosage form. The pH gradient in humans begins with a pH of 1 to 2 in the stomach, followed by a broader pH range of 5 to 8 in the small intestine, with the intermediate range of pH values of around 5 to 6 being found in the duodenum. Colonic absorption in the last segment of the GI Tract occurs in an environment with a pH of 7 to 8. The average pH values significantly differ between the fed and the fasted state. Finally, gastric emptying time and intestinal transit time are very important for drug absorption. The majority of the liquid gets emptied from the stomach within 1 hour of administration (Washington *et al.*, 2001). Food and other solid materials, on the other hand, take 2 to 3 hours for half of the content to be emptied. The general range of the small intestinal transit time does not differ greatly and usually ranges from 3 to 4 hours (Davis *et al.*, 1986).

2.3.6 Physicochemical properties

2. 3. 6 .1 Solubility

The aqueous solubility of a drug substance is a fundamental property that should be evaluated early in discovery. Lack of solubility can affect the results of early high-throughput screening assays and the ability to achieve efficacious and toxicologically relevant exposures in animals. This characteristic will also affect the future developability of and formulation efforts for the compound. Solubility depends on the solvation energy of the solute in the solvent overcoming both the crystal lattice energy of the solid and the energy to create space in the solvent for the solute. Thus, the solubility of a compound depends not only on properties of the drug molecule itself, such as polarity, lipophilicity, ionization potential, and size, but also on properties of the solvent and the solid, such as the crystal packing and presence of solvates (Florence, 1998).

Two main types of methods for the determination of drug solubility: (i) Kinetic methods that estimates the non equilibrium solubility in a high throughput mode and (ii) Thermodynamic methods that estimate the solubility at equilibrium. These are examples of methods with a high capacity (50 - 300 compounds per day) used by pharmaceutical companies for fast estimation of the solubility in buffer provided by turbidimetric and the nephelometric methods. The advantage of using these methods is their speed and the fact that they estimate the solubility under conditions similar to those used in biological assays (Lipinski *et al.*, 1997; Bevan and Lloyd, 2000).

Thermodynamic methods result in intrinsic solubility, since solubility is measured at equilibrium between the solid and the saturated solution. The most commonly used method is the shake-flask method (Glomme *et al*., 2005; Bergström *et al*., 2002).

2.3.6.1.1 Factors influencing solubility experiments

When determining solubility, very different values may be obtained depending on the experimental set -up and the methods used. Drugs often contain ionisable groups such as amines, carboxyl acids and sulphonamides. The ratio of ionized and unionized compound will vary with the pH of the solution, depending on whether the protolytic function is an acid or a base and on the pK_a of that group the ionized form has a higher solubility than does the unionized form.

The physical form of the solid will influence the solubility, salts, solvates and polymorphs of the same compound exhibit different solubilities. For some compound this effect can be large, while it is less pronounced for others. Typically, the difference in solubility between two polymorphs of the same compound is around a factor of two (Pudipeddi and Serajuddin, 2005). There are several other aspects related to the physical form that will influence the solubility; the crystalline form of a compound is generally less soluble than the amorphous form of the same compound. Hydrates usually exhibit a lower solubility in water than their corresponding anhydrous form. When dissolved, any metastable form will transform into the thermodymamically stable form at the relevant temperature and pressure. Furthermore, the purity of the compound influences the

solubility, with this effect being more pronounced for poorly soluble compound (Grant and Higuchi, 1990).

The solubility of a salt will decrease upon the addition of a common ion due to a shift in equilibrium in favour of solid state of the salt. Oppositely, addition of non-common ion will result in an increased solubility of a sparingly soluble salt. For non-electrolytes, on the other hand, an increase in the ionic strength will result in a decrease in the solubility. Dissolution in water is an endothermic process for most compounds. This results in an increase in the equilibrium solubility with an increase in temperature. The addition of a co-solvent (a water–miscible organic solvent) will increase the aqueous solubility of hydrophobic compounds; the larger and more non–polar the solute the greater the effect of the co-solvent. For this reason, co-solvent is often used to facilitate the determination of poorly soluble compounds or when determining solubility by automated methods starting from dimethyl sulfoxide (DMSO) stock solution using co-solvent will change the experimental conditions, resulting in a different solubility value than if pure water and/or solid compound had been used (Wassvik, 2006).

2.3.6.2 Dissociation Constant (pKa)

The p*K*a, or dissociation constant, is a measure of the strength of an acid or a base. The dissociation constant of an organic acid or base is defined by the following equilibria:

$$HX + H_2O \rightleftharpoons H_3O^+ + X^- \quad \text{(acid)} \quad \text{.....(2)}$$

$$BH^+ + H_2O \rightleftharpoons H_3O^+ + B \quad \text{(base)} \quad \text{.....(3)}$$

$$K_a = \frac{[H_3O^+][X^-]}{[HX]} \quad \text{(acid)} \quad \text{.....(4)}$$

$$K_b = \frac{[H_3O^+][B]}{[HB^+]} \quad \text{(base)} \quad \text{.....(5)}$$

Whereby the log *K*a is defined as p*K*a. The p*K*a of a base is actually that of its conjugate acid. As the numeric value of the dissociation constant increases (i.e., p*K*a decreases), the acid strength increases. Conversely, as the acid dissociation constant of a base (that of its conjugate acid) increases, the strength of the base decreases. For a more accurate definition of dissociation constants, each concentration term must be replaced by thermodynamic activity. In dilute solutions, concentration of each species is taken to be equal to activity. Activity-based dissociation constants are true equilibrium constants and depend only on temperature.

Most p*K*a values in the pharmaceutical literature are measured by ignoring activity effects and therefore are actually concentration dissociation constants or apparent dissociation constants. It is customary to report dissociation constant values at 25°C

(Smith and O' donell, 2006). Drug dissociation constants are experimentally determined by manual or automated potentiometric titration or by spectrophotometric methods (Avdeef, 2001). Current methods allow determination of p*K*a values with drug concentrations as low as 10 to 100 µM. For highly insoluble compounds (concentration 1 to 10 µM), the Yesuda–Shedlovsky method (Avdeef *et al*., 1999) is commonly used where organic co-solvents such as methanol are employed to improve solubility. The method takes three or more titrations at different co solvent concentrations, and the result is then extrapolated to pure aqueous system.

For a compound containing basic or acidic functional groups, solubility at a given pH is influenced by the compound's ionization characteristics. The solubility of a compound in aqueous media is greater in the ionized state than in the neutral state. Thus, solubility of ionizable compounds is dependent on the pH of the solution. Many drugs are weak acids or bases and thus are ionizable within the pH range of the gut. Solubility and dissolution, and therefore, absorption, of a weak base can be altered by changes to gastric pH for example when co-administered with ant-acids. While a weakly basic compound might fully dissolve in the acidic environment of the stomach and result in high exposure levels under such conditions, co- administration of drugs that raise the stomach pH can lead to greatly decreased solubility, leading to significantly lower exposure (Smith and O' donell, 2006) .

Other considerations for an ionizable compound include the impact of ionization on stability and permeability and for compounds containing both acidic and basic functional

groups the formation of zwitterions, for which the solubility at the isoelectric point is typically the lowest over the entire pH range. If a compound is ionisable, a salt can be formed, which may have better dissolution or an improvement in other physical properties such as hygroscopicity, solid-state stability, or the potential for polymorphism. There are many experimental methods used in a discovery setting for determining the pKa. Simple fitting of the pH solubility profile can be used if solubility measurements have already been made at multiple pH values. Other typical methods include potentiometric titration, spectrophotometric titration, and capillary electrophoresis (Smith and O' donell, 2006).

The characteristic thermodynamic parameter relating the pH to the charge state of a molecule is the ionization constant PK_a (Perea *et al*., 1991). Knowledge of the pK_a of a substance is widely useful as it can predict the absorption, distribution and elimination of medicinal substances in a biological system.

2.3.6.2.1 Determination of pKa of poorly soluble drugs

There are various methods for measuring pKa values but potentionmetry has out standing importance because it is fast, accurate and reproducible (Albert and Serjeant, 1984).

However, the application of pH-metric pKa measurement is often hindered by the poor water solubility of the sample. By using a glass electrode of excellent quality,

performing proper electrode calibration, excluding the presence of a ambient carbon dioxide as much as possible and accurately dispensing very small titrant volumes, potentiometry in aqueous solution can be applied to concentration as low as 10^{-4} M. The accuracy and reproducibility of titration in such dilute solutions is much less than in higher concentration. The best tool of pKa determination in very dilute (10^{-5} to 10^{-6} M) solutions is spectrophotometry, provided that the compound posses pH-dependant UV absorption due to the presence of of a chromophore in proximity to the ionizable group (Albert and serjeant, 1984). However if the molecule suffers from both problems of very poor solubility and lack of an analytical useful chromophore, alternative methods of pKa determination such as mixed –solvent procedure can be used (Mizutani ,1925).

This method is based on the measurement of apparent ionization constants ($p_o ka$) in different ratios of organic solvent/water mixtures where the aqueous pKa is obtained by extrapolation. Organic solvents frequently used are methanol, ethanol, propanol, DMSO, DMFA, acetone and THF. Since most literature data has been accumulated for MeOH/water solvent mixtures and since it is generally accepted that methanol shows a solvation effect closest to water, it is normally chosen as the organic solvent of choice (Avdeef *et al*., 1993).

2.3.6.2.2 SUPERQUAD and MOLSPIN pH auto-titration

MOLSPIN program is high precision input software designed to be driven by compatible computer. MOLSPIN pH meter software is capable of working in conjunction with SUPERQUAD program. The power and flexibility of the MOLSPIN

pH meter can be readily applied because the control software is held in the disk and can be modified as required. The titration option provides the most powerful facilities. Auto titrations can be performed using 1 or 2 electrodes with measurements in either mV or pH. The total volume to be delivered and increment size is under user control. The user can skip a reading or terminate a titration at any time. Progress of a titration is monitored in both tabular (volume - pH/mV) and graphical form. When a titration is complete the data can be saved in the disk using either text or in SUPERQUAD format.

In this study, potentiometric data collected using a MOLSPIN pH meter was calculated by SUPERQUAD program to ionization constants. The end point of a potentiometric titrations were found by plotting pH as a function of volume (v) of the reagent added in a Gran plot which is common means of standardizing a titrate or a titrant by estimating the equivalence volume or end point in a potentiometric titration. The end point was the point of inflexion of the curve.

2.3.6.3 Partition and distribution coefficient (Log P and Log D)

In the fields of organic and medicinal chemistry, a partition (P) or distribution coefficient (D) is the ratio of concentrations of a compound in the two phases of a mixture of two immiscible solvents at equilibrium (Leo *et al.*, 1971). Hence these coefficients are a measure of differential solubility of the compound between these two solvents. Normally one of the solvents chosen is water while the second is a hydrophobic such as octanol (Sangster, 1997). Hence both the partition and distribution coefficient are measures of how hydrophilic ("water loving") or hydrophobic ("water

hating") a chemical substance is. Partition coefficients are useful for example in estimating distribution of drugs within the body. Hydrophobic drugs with high partition coefficients are preferentially distributed to hydrophobic compartments such as lipid bilayers of cells while hydrophilic drugs (low partition coefficients) preferentially are found in hydrophilic compartments such as blood serum.

The partition coefficient (P) is a measure of how a drug partitions between a water-immiscible lipid or an organic phase and water. It is defined as follows:

$$P = \frac{[\text{Neutral species}]_o}{[\text{Neutral species}]_w} \qquad \text{.....(6)}$$

$$\text{Log } P = \text{Log}_{10} P \qquad \text{.....(7)}$$

The distribution coefficient is the partition coefficient at a particular pH. The following equilibrium is often used to define *D*, with the assumption that only the unionized species

partition into the oil or lipid phase:

$$D = \frac{\left[\text{Unionized species}\right]_o}{\left[\begin{matrix}\text{Unionized}\\ \text{species}\end{matrix}\right]_w + \left[\begin{matrix}\text{Ionized}\\ \text{species}\end{matrix}\right]_w} \quad \text{.....(9)}$$

$$\text{Log } D = \begin{cases} \text{Log p - Log } \left[1 + 10^{(pH - pk)}\right] & \text{acids} \\ \text{Log P- Log } \left[1 + 10^{(pK - pH)}\right] & \text{bases} \end{cases} \quad \text{.....(10)}$$

Log D is pH dependent, hence one must specify the pH at which the log D was measured. Of particular interest is the log D at pH = 7.4 (the physiological pH of blood serum). For un-ionizable compounds, log P = log D at any pH. The measurement of log *P* is important because it has been shown to correlate with biological activity and toxicity (Hansch and Leo, 1979).

2.3.6.3.1 Measurement of log P

The methods commonly used for the measurement of log p include traditional shake-flask method, HPLC, filter- probe and pH metric techniques. The shake flask method is the most commonly used method for the measurement of log P (Leo, 1997). However, for many insoluble compounds, the solubility in the aqueous phase may be too low to be accurately determined. In these cases, alternative method needs to be applied.

The determination of partition coefficients by the potentiometric method is part of the function of the Sirus PCA 101 (Avdeef, 1993). Typically a pre acidified solution of a weak acid is alkametrically titrated to some appropriately high pH then the partition

solvent such as octanol is then added and the dual- solvent mixture is acidimetrically titrated back to the starting pH. Analysis of the two titration curves will yield two pK_as; pK_a and p_oK_a where p_oK_a is the apparent constant derived from the octanol-containing segment of data. The partition coefficient is calculated from the following equations:

for an acid: $P_{HA} = (10^{(p_oKa - PKa)} - 1) / r$ ……. (11)

for a base : $P_{HA} = (10^{-(p_oKa - PKa)} - 1) / r$ ……. (12)

Where r = $\frac{\text{Volume of the organic base}}{\text{Volume of the aqueous base}}$

2.3.6.4 Log P / Log D and biological activity

Drugs exert their therapeutic effects through reactions with specific receptors. Drug-receptor binding depends on the concentration of the drug near the receptor. Its form and concentration near the receptor depend on its physicochemical properties. Orally administered drugs need to be dissolved at the site of absorption in the gastrointestinal tract (GIT) and need to traverse several membrane barriers before receptor interactions can commence. As the drug distributes into the various compartments of the body, a certain portion finds itself in the receptor site (Avdeef, 2001).

In the context of pharmacokinetics (what the body does to a drug), the distribution coefficient has a strong influence on ADME properties (Absorption, Distribution, Metabolism, and Excretion) of the drug. Hence the hydrophobicity of a compound (as

measured by its distribution coefficient) is a major determinant of how drug-like it is. More specifically, in order for a drug to be orally absorbed, it normally must first pass through lipid bilayers in the intestinal epithelium (a process known as transcellular transport). For efficient transport, the drug must be hydrophobic enough to partition into the lipid bilayer, but not so hydrophobic, that once it is in the bilayer, it will not partition out again. Likewise, hydrophobicity plays a major role in determining where drugs are distributed within the body after adsorption and as a consequence in how rapidly they are metabolized and excreted (Kubinyi, 1979).

In the context of pharmacodynamics (what a drug does to the body), the hydrophobic effect is the major driving force for the binding of drugs to their receptor targets (Eisenberg and Melachlan, 1986; Miyamoto and Kollman, 1993). On the other hand, hydrophobic drugs tend to be more toxic because they in general are retained longer, have a wider distribution within the body (*e.g.*, intracellular), are somewhat less selective in their binding to proteins, and finally are often extensively metabolized. In some cases the metabolites may be chemically reactive.

Hence it is advisable to make the drug as hydrophilic as possible while it still retains adequate binding affinity to the therapeutic protein target (Pliska *et al.*, 1996). Therefore, the ideal distribution coefficient for a drug is usually intermediate (not too hydrophobic or too hydrophilic).

Table 1. Literature values of Log D and their implications for Drug development

Log D	**Implications for Drug development**
Below 0	Intestinal and CNS permeability problems Susceptible to renal clearance
0 – 1	May show a good balance between permeability and solubility
1 – 3	Optimum range for CNS and non-CNS orally active drugs Low metabolic liabilities, generally good CNS penetration
3 – 5	Solubility tends to become lower and metabolic liabilities increase.
Above 5	Low solubility and poor oral bioavailability. Erratic absorption, high metabolic liabilities although potency may still be high

Source: Shalaeva, 2002

Table 2. Literature values of optimum Log P at various absorption sites

Absorption site	Log P (Optimum)
CNS penetration	2 +/- 0.7
Oral absorption	1.8
Intestinal absorption	1.35
Colonic absorption	1.32
Sub lingual absorption	5.5
Percutaneous	2.6

Source: Earl, 1999

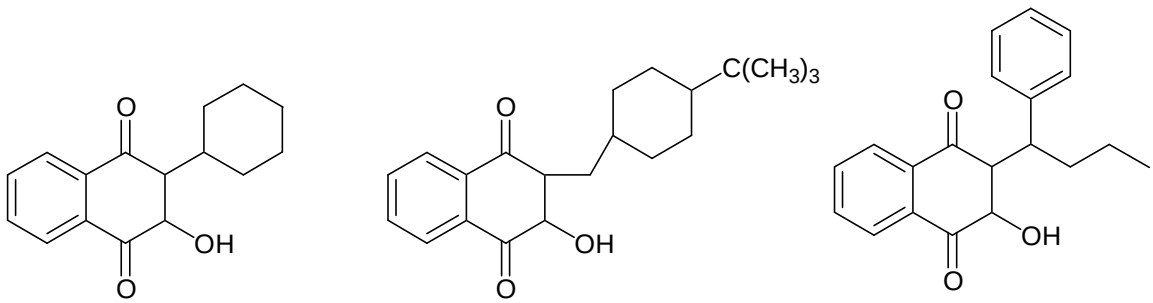
Drugs should be designed with the lowest possible log P to reduce toxicity, non specific binding, increase ease of formulation and bioavailability. Drugs should also be as low molecular weight as possible to lower the risk of allergic reactions (Earl, 1999).

2. 4 Parvaquone and Buparvaquone

Parvaquone (**16**)**,** has structural similarities with buparvaquone **(17)**, as well as 2-Hydroxy-1,4-Naphthoquinone(**12**) and 2-Hydroxy-3-(2-Phenylbutyl)-1,4-Naphthoquinone (**18**) all being hydroxy napthoquinones, the difference occurring in the substituents on the side chains. Parvaquone and buparvaquone (Fig. 7) are currently being used in the management of East Coast fever (ECF), a protozoa infection, in cattle

(Muraguri *et a*l., 1999). Buparvaquone has shown high activity *in vitro* against *L. donovani* amastigotes however, the *in vivo* activity of buparvaquone against *L. donovani* was low after subcutaneous administration. The low aqueous solubility and lipophilicity of buparvaquone leads to poor distribution from the site of injection to the intracellular target and low oral bioavailability (Croft *et al.*, 1992).

Figure 7 . Commercial packages of Parvexon (parvaquone) and Butalex (Buparvaquone).



16 **17** **18**

In the present study parvaquone and related compounds, **12, 17** and **18** in the class of napthoquinones will be assessed for their solubility, pKa, logP and *in vitro* anti-

leishmania activity. The compound(s) with the most favorable physicochemical parameters and *in vitro* activity will be identified.

2. 5 Rationale and justification

The current drugs used for the management of Leishmaniasis include pentavalent antimony (Sb^{v}) or amphotericin B that can only be administered parenterally. This means long hospital stay and the presence of skilled health personnel to administer treatment throughout duration of therapy. Resistance to pentavalent antimony has been reported (Berman, 1997)) and visceral leismaniasis is emerging as an important opportunistic infection among HIV positive patients. Thus a real need exists for improved anti-leishmanial drugs particularly for oral administration in the treatment of VL and for topical administration in the treatment of cutaneous leishmaniasis (CL).

The population in the endemic countries cannot afford the newer formulations of amphotericin B, which are more effective and less toxic. The new drugs should therefore be affordable to the target population. One way of achieving this is to screen existing drug molecules that are currently used in the management of other disease conditions for anti-leishmania activity. Those that show strong activity can be optimized through Quantitative Structure Activity Relationship (QSAR) to produce efficacious drug molecules. Parvaquone is not a novel compound but a well-known hydroxynapthoquinone used in the management of theilerosis in cattle. In this study, we propose to carry out physicochemical as well as *in vitro* evaluation for parvaquone and

related compounds with a view to identifying the most suitable compound that can be further developed for oral delivery.

2.6 Statement of the Problem

Leishmaniasis is regarded as among the neglected diseases where there is little research and development for new, safe and efficacious drugs. The existing first line treatment for the disease was developed over 40 years ago and can only be administered parenterally with long hospital stay (Mantyla *et al.,* 2004). This translates to high treatment costs. Leishmaniasis is also emerging as an important opportunistic infection among HIV positive patients. Resistance to the current treatment, pentavalent antimonials, has been reported. The newer formulation of amphotericin B which is more effective and less toxic is too expensive for the majority of the affected population. There is a real need to develop new affordable anti-leishmanial drugs.

2. 7 Hypothesis

2.7. 1 Alternative Hypothesis

Parvaquone and related compounds have good *in vitro* anti-leishmanial activity and unfavourable physicochemical profiles for oral administration.

2.7. 2 Null Hypothesis

Parvaquone and related compounds have no *in vitro* anti-leishmanial activity

2. 8 Objectives

2. 8. 1 General objectives

To determine the physicochemical profiles and *in vitro* activity of parvaquone and related compounds.

2.8.2 Specific objectives

i) To determine the pKa and log P of Parvaquone**,** Buparvaquone, 2-Hydroxy-1, 4-Naphthoquinone and 2-Hydroxy-3-(2-Phenylbutyl)-1, 4- Naphthoquinone.

ii) To determine the anti-leishmanial activity of Parvaquone**,** Buparvaquone, 2-Hydroxy-1, 4-Naphthoquinone and 2-Hydroxy-3-(2-Phenylbutyl)-1, 4-Naphthoquinone in *vitro*.

iii) To measure the immuno-stimulatory activity of Parvaquone**,** Buparvaquone, 2-Hydroxy-1,4-Naphthoquinone and 2-Hydroxy-3-(2-Phenylbutyl)-1, 4-Naphthoquinone .

iv) To determine cytotoxicity of Parvaquone**,** Buparvaquone, 2-Hydroxy-1, 4-Naphthoquinone and 2-Hydroxy-3-(2-Phenylbutyl)-1, 4- Naphthoquinone.

CHAPTER 3 : MATERIALS AND METHODOLOGY

3.1 Study Design

The solubility, pKa and Log P of parvaquone and related compounds were determined following literature methods for poorly soluble compounds. The anti-leishmania activity of parvaquone and related compounds was determined through *in vitro* assays using *Leishmania major* (strain NLB 144*)* and *Leishmania donovani* parasites in cell free culture and in infected macrophages. Cell free cultures were done in Complete Schneider's Insect Medium (CSIM) supplemented with Fetal Bovine Serum (FBS) at 25^{o} C. The viability of promastigotes was determined using Trypan blue stain exclusion principle. Cells were counted using an improved Neubauer chamber. The supernatants from control and Leishmania infected macrophages were analyzed for their nitrite content by Griess reaction in order to determine their immuno- stimulative effect (Holzmuller *et al.*, 2002). Absorbance was measured at 550 nm and the concentration of nitrite calculated using a linear regression of a standard curve.

All experiments were done in triplicates and the average of the three sets of results computed. In the determination of pKa and Log P an MOLSPIN pH auto titration programme was used to carry out experiment and collect data which was then used with the aid of SUPERSQUAD programme to compute the pKa of the compounds under test.

3.2 Determination of physicochemical parameters

The determination of aqueous solubility was done at the Quality Control laboratory of the Production Department, KEMRI while pKa and Log P measurements were done at Research laboratory of the Chemistry Department, Kenyatta University.

3.2.1 Determination of aqueous solubility

The kinetic method of aqueous solubility measurements as described by (Wassvik, 2006) and Lipinski et al., 1996 was used.

Briefly, 0.004 g of compound under test was weighed and dissolved in 400 µl of Dimethyl Sulfoxide (DMSO). Then 7.5 µl of this solution was aliquoted into a mixing tube and made up to 5 ml using pH 7 phosphate buffer solution and vortexed to achieve complete mixing. This was labeled sample 1 and starting with this sample serial dilution by a factor of 2 was done with a final DMSO solution having a concentration of 0.0015 µg/ml. The absorbance of each dilution was measured at 650 nm using a UV spectrophotometer. The UV measurement was repeated three times and the average absorbance computed. Precipitation was detected as an increase in UV absorbance by light scattering at 650 nm.

This was repeated using a phosphate buffer pH 4 for each of the compounds.

3.2.2 Determination of ionization constant

3.2.2.1 Standardization of 0.001M NaOH

3 g of Potassium Hydrogen Phthalate (KHP) was carefully weighed. It was then oven dried at 110°C for at least one hour. KHP solid was then cooled in a desiccator for about 15 min to avoid substantial errors in further measurements. Three samples of KHP weighing 0.0512 g, 0.0509 g and 0.0520 g were each dissolved in 25 ml distilled water. The solutions were poured in 250 ml conical flasks; two to three drops of

phenolphthalein indicator were added in each flask. NaOH solution was filled in 50 ml burette and titrated against the KHP solution. The results were recorded and further used in calculation of exact concentration of NaOH solution.

3.2.2.2 Automated titration system

The arrangement for the potentiometric titration is shown in the set up and in the scheme in Fig. 8 and 9 . It consisted of an IBM compatible PC, micro-syringe, titration vessel (1-5 ml), the MOLSPIN pH auto titration programmer and a combined glass-calomel electrode. The MOLSPIN programme has a facility to measure pH with automatic temperature compensation.

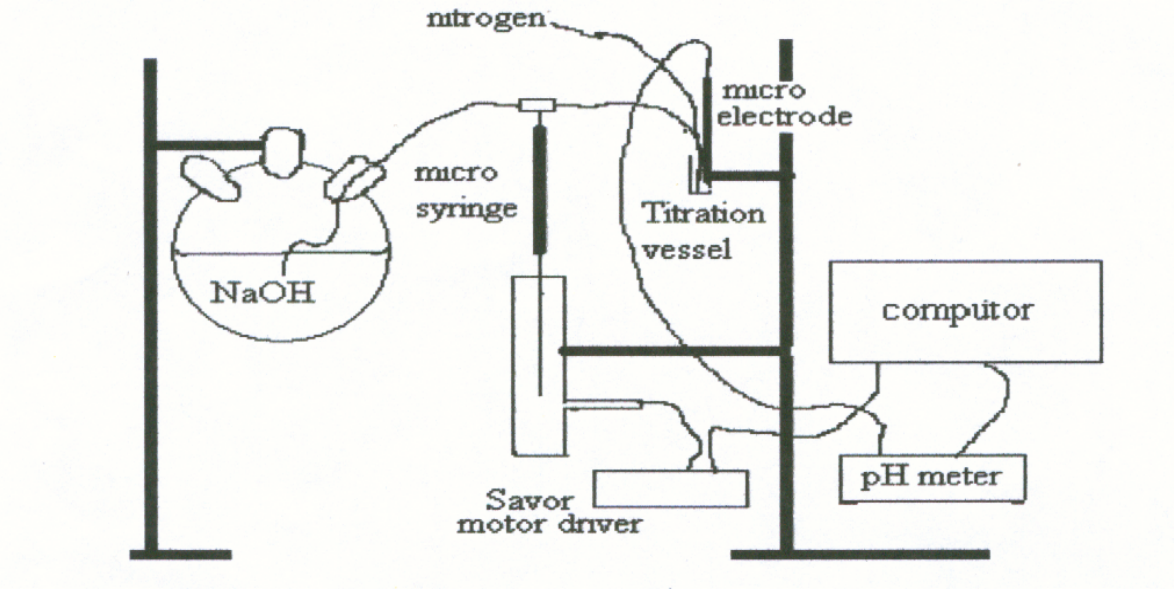


Figure 8. Diagram of the experimental set up

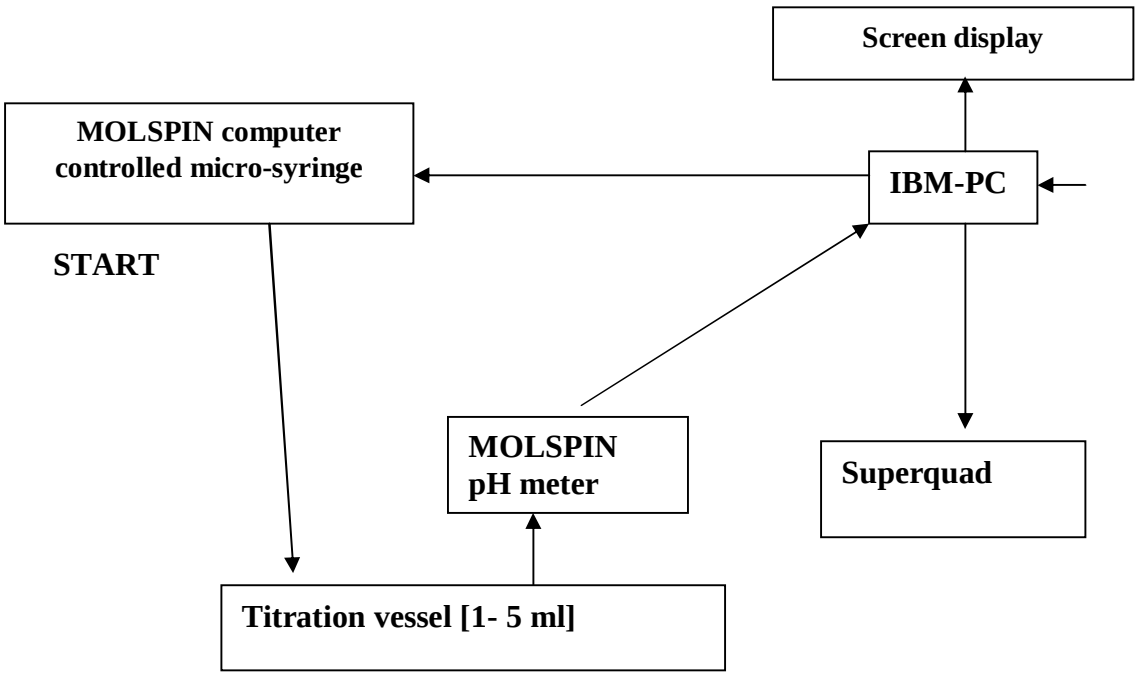


Figure 9. The schematic arrangement of the experiment.

The compounds under study (10 mg each) was dissolved in three different methanol/water mixtures of 30, 40 and 50 (vol %) in 0.15M KCl and consequently three separate titrations were performed. The maximum titrant volume increment for one titration step was limited to 0.01 ml. The reactions were carried out under constant temperature 25^0C in inert conditions over a pH range of 3 to 14. A total of 102 pH readings were collected from each titration using the automated titration system described above. Processing of three separate titration data and the extrapolation of the

aqueous pK_a based on the Yasuda-Shedlovsky approach was done (Mantyla *et al*., 2004 and Takacs-Novak, 1997).

According to this method a linear correlation is established in a plot:

$$psKa + \text{Log}\ [H_2O] \quad \text{Versus}\ a/\varepsilon + b$$

Where Log $[H_2O]$ is the molar water concentration of a given solvent, ε is the dielectric constant of the mixture and a and b are the slope and intercept respectively. Using simultaneous equations the values of a and b was calculated and thereafter aqueous pKa values were obtained for log 55.5 and 1/78.3 the molar concentration and dielectric constant of pure water.

3.2.3 Determination of log P and log D

Parvaquone (10 mg) and related compounds were each dissolved in 5 ml Octanol to make stock solution. Using the recommended volume ratio of octanol/water for each of the compounds water was added to volume (OECD guideline 122, 2000). Automatic titration was done as described above. This was repeated three times for each of the compounds and p_oKa recorded.

Using the pKa obtained above and the p_oKa of each of the compounds the log P can then be computed using equation 13;

$$P = (10^{(p_oKa\ -\ PKa)} - 1) / r \ \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots. \ (13)$$

Where r = Volume of the organic base

Volume of the aqueous base

p_oKa and pKa are apparent ionization constant in octanol/water and in aquase solutions respectively. Log P will then be obtained from the P value.

Distribution coefficient is related to log p by the following equation applied to acidic substances:

$$\text{Log D} = \text{Log P} - \text{Log } (1 + 10^{(pH-pKa)}) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (14)$$

Where:

D = Distribution coefficient

P = Partition coefficient

$_p$ka = Ionization constant

Hence using equation 14 log D will be determined.

3.3 *In vitro* Tests

The *in vitro* assays were done at the leishmaniasis laboratory at the Centre for Biotechnology Research and Development, KEMRI as detailed below.

3.3.1 *Leishmania major* Parasites

Leishmania major parasites (Strain IDU/KE/83 = NLB-144) was used in this study. These parasites were originally isolated from a female *Phlebotomus duboscqi* and maintained by cryospreservation, *in vitro* culture, and periodic passage in BALB/c mice

at CBRD, KEMRI. *Leishmania major* promastigotes were cultured in NNN media overlaid with 2 ml Schneider's *Drosophila* medium, supplemented with 20% fetal bovine serum (FBS), 100 ug/ml streptomycin and 100 U/ml G-penicilin, and 5 fluorocytosine. The cultures were made in sterile disposable culture flasks (25 cm^3) and incubated at

25^o C as recommended by Evans *et al.*, (1989), to the stationary metacyclic stage.

3.3.2 Test for anti-leishmanial activity in cell free culture

A stock solution of 1,000 µg/ml of each of the drugs including the controls was prepared. Using 24 well plates serial dilution were made in triplicates with the final concentration being 12.5 µg/ml. In to each well plate 1X 10 ^{6} of parasites were added from the parent culture and the plates then sealed. The preparation was incubated at 25º C – 28 º C for 48 hours. The motility of parasites were observed using inverted microscopes and concentration at which motility stops were marked as minimum inhibitory concentration

(MIC). There after a 100 µl of the preparations from each concentration were transferred to a 96 well plate in their respective order and 10 µl of 10mg/ml MTT was added. They were then incubated in the dark for four hours. The formazan produced were dissolved by adding 100 µl of DMSO and the optical density determined using ELISA reader at 540 nm.

3.3.3 Test of drugs on infected macrophages

3.3.3.1 Harvesting of macrophages

This was done using literature methods (Tonui and Titus, 2007). Briefly, BALB/c mice were injected with 2% starch solution to stimulate macrophage proliferation, and later sacrificed after 24 hr. The body surface was disinfected, and the skin torn dorso-ventrally to expose the peritoneum. A sterile syringe was used to inject (10 ml) of chilled incomplete RPMI 1640 into the stimulated peritoneum. Mouse peritoneal macrophages were harvested by withdrawing the fluid and placing it in sterile centrifuge tubes. The cell suspension were centrifuged at 5,000 rotations per min at 4° C for 19 min and the pellet resuspended in (5 ml) complete RPMI 1640 medium.

3.3.3.2 Activity of parvaquone and the pro-drugs

Macrophages were absorbed in 24-well plates and allowed to adhere for 4 hr at 37^{o}C in 5% CO_2. Non-adherent cells were washed with PBS, and the macrophages incubated overnight in RPMI 1640 media. Adherent macrophages were infected with *L. major* promastigotes at a parasite/macrophage ratio of 6:1, incubated at 37^{o}C in 5% CO_2 for 4 hr, free promastigotes removed by extensive washing with HBSS, and the cultures incubated in RPMI 1640 medium for 24 hr. Treatment of infected macrophages with parvaquone and related compounds was done once. Pentostam® was used as a positive control for parasite growth inhibition. The medium and drug were replenished daily for 3 days. After 5 days the monolayers were washed with PBS at 37^{o}C, fixed in methanol

and stained with Giemsa solution. The number of amastigotes was determined by counting at least 400 macrophages in triplicate cultures and results expressed as infection rate (IR) and multiplication index (MI) (Berman and Lee, 1983).

IR = No. of infected macrophages in 100 macrophages

MI = (No. of amastigotes in experimental culture/100 macrophages) x 100 %

(No. of amastigotes in control culture/100 macrophages) …….

(15)

3.3.4. Measurement of nitric oxide production

Nitric oxide release in supernatants of macrophage culture was measured by the Griess reaction for nitrites as described by Holzmuller *et al*., (2002). Protective immunity depends on the activation of macrophages to kill the parasite though the nitric oxide mediated mechanism. Briefly, supernatants were collected (100 ul), 48 hr after introducing Parvaquone into the culture medium. This was put in triplicate wells in a 96 well microtiter plate. To this, 60 ul of Griess Reagent A (1% sulfanilamide in 1.2 M HCI) was added, then 60 ul of Griess Reagent B [0.3% N- (-aphthyl]ethylenediamine)]. The plates were read at 540 nm in an enzyme-linked immunosorbent assay (ELISA) plate reader. Sodium nitrate ($NaNO_2$) in RPMI was used to construct a standard curve for each plate reading. The procedure was repeated for the other compounds under test.

3.3.5 Cytoxicity assay

A 100 µl of cell suspension was added to 96 well plates in triplicate and incubated overnight at 37°C, 5 % CO_2. The spent media was discarded and drugs under test were added at varying concentration between 31.25 µg/ml and 1000 µg/ml. It was then incubated for 48 hrs under initial conditions. The cells were then checked under inverted microscope to ensure normal growth. The used media were discarded and 50 µl of 10 mg /ml MTT was added. It was then incubated at 37°C for 4 hr and 100 µl of DMSO was added and optical density read at 540 nm.

The percent viability of cells was computed as follows;

$$\% \text{ Viability} = \frac{\text{Absorbance of treated cells - Absorbance of blank X 100}}{\text{Absorbance of control cells – Absorbance of blank}} \quad \ldots\ldots\ldots (16)$$

A dose response curve of % cell viability versus drug concentration was drawn and LC_{50} which is drug concentration at 50% cell viability was identified for each compound from the curves and recorded. Using LC_{50} and the MIC earlier obtained the Selectivity index (SI) for each compound under test was computed as follows;

$$\text{Selectivity Index (SI)} = \frac{\text{Lethal Concentration } (LC_{50})}{\text{Minimum Inhibitory Concentration (MIC)}} \quad \ldots\ldots\ldots\ldots (17)$$

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1 Physicochemical measurements

4.1.1 Aqueous solubility measurements

The aqueous solubility of the compounds under test at pH 4 and 7 are summarized in Table 3 below;

Table 3. Aqueous solubility of the test compounds at pH 4 and 7

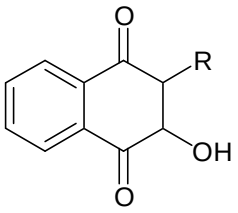
Compound	Aqueous solubility µg/ml	
	pH 4	pH 7
NQ	1.875	> 15
Parvaquone	0.014	0.937
Buparvaquone	0.007	0.029
PNQ	0.029	0.014

Key :(i) NQ – 2-Hydroxy -1, 4- Napthoquinone (ii) PNQ – 2-Hydroxy-3-(2- Phenylbutyl)-1, 4- Naphthoquinone

The compounds generally exhibit poor aqueous solubility with the NQ having the highest solubility among the test compounds at > 15 µg/ml at pH 7. Buparvaquone had the lowest solubility of 0.007µg/ml at pH 4. All the compounds except PNQ had higher solubility at pH 7 than at pH 4, this is due to the fact that they are weak acids and there is ionization at higher pH. Solubility is highest when the substituent R is a Hydrogen atom, however when R is an alkyl side chain there is a substantial decrease in solubility

as shown by the results above regardless of the pH with which it was measured. Parvaquone and Buparvaquone both have cyclohexyl group in the side chain, the difference being tertbutyl group present in Buparvaquone. The presence of tertbutyl group in Buparvaquone side chain seems to decrease the solubility as later has a lower solubility than parvaquone.

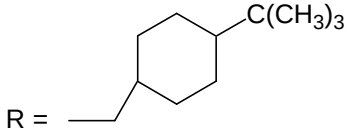
From the results, the aqueous solubility of PNQ seems to be decreasing with increase in pH unlike the rest of the compounds. The presence of a phenyl group in the side chain in PNQ which the other compounds do not have might be inhibiting the ionization of this compound at higher pH.



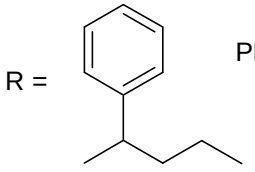
Napthoquinone moiety

R = H NQ

R = Parvaquone



R = Buparvaquone



R = PNQ

The pH of the stomach varies between 1 – 4 hence investigating the solubility at pH 4 gives an indication of the compound behavior in the acidic environment of the stomach if it is to be delivered orally. The pH of plasma and interstitial fluids is 7.4 (Mantyla *et al*., 2004) therefore determining the solubility of compounds at this pH will give an indication of their behavior once delivered into body fluids such the circulatory system or muscle tissue.

According to literature (Lipinski *et al.*, 1997), most drugs that are orally active have an aqueous solubility greater than 65µg/ml but when the solubility is 20 µg/ml or less the probability of useful oral activity is very low unless the compound is unusually potent. This is due to low bioavailability associated with poor solubility. The compounds under test have low aqueous solubility and therefore they might exhibit poor oral activity.

4.1.2 Ionization constants (pKa) and Partition coefficient (log P) measurements

pKa and log P are summarized in the Table 4 and Figure 10 below ;

Table 4. pKa and Log P of compounds under test

Drug	pKa	Log P
NQ	3.99	6.98
Parvaquone	4	4.21
Buparvaquone	6.58	2.64
PNQ	8.68	9.79

Key: (i) NQ – 2-Hydroxy -1, 4- Napthoquinone (ii) PNQ - 2-Hydroxy-3 -(2-Phenylbutyl)-1, 4- Naphthoquinone

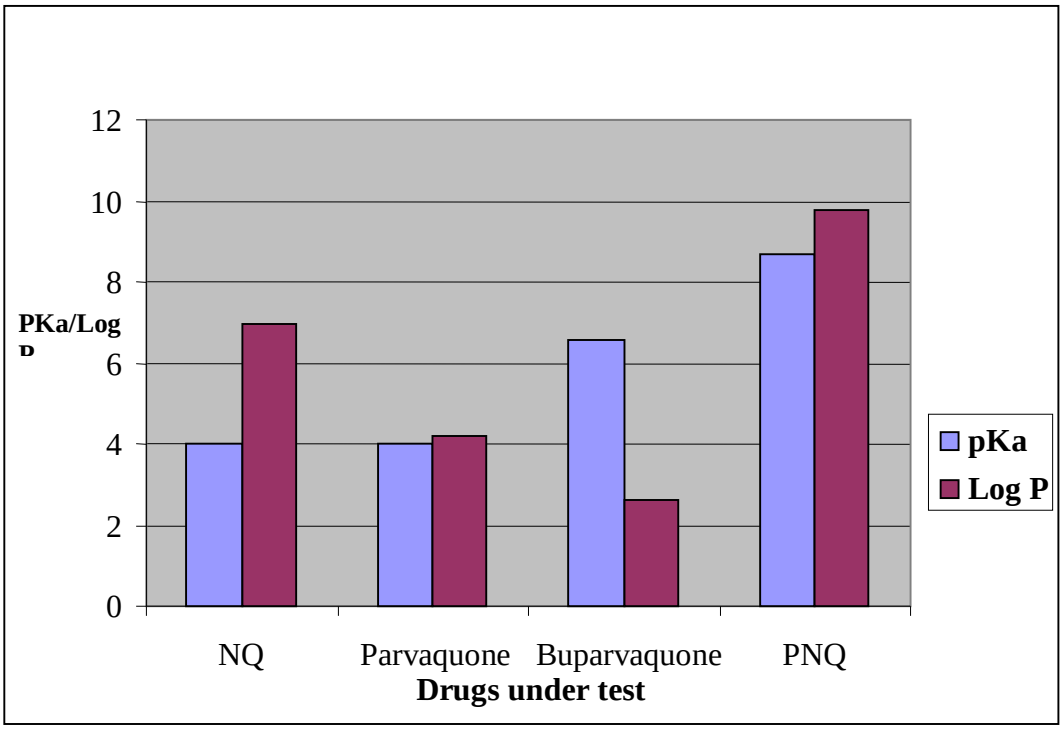


Figure 10. **The pKa and Log P values of drugs under test**

Key: (i) NQ – 2-Hydroxy -1, 4- Napthoquinone (ii) PNQ - 2-Hydroxy-3-(2-Phenylbutyl)-1, 4-Naphthoquinone (iii) PVQ – Parvaquone (iv) BPVQ- Buparvaquone

NQ has the lowest pKa among the test compounds and PNQ had the highest. Looking at the R substituents NQ has H while Parvaquone, Buparvaquone and PNQ have cyclohexane, tertbutylcyclohexane and phenylbutyl groups respectively. The results show that the bigger the side chain (higher molecular weight) the higher the pKa values. This means that alkyl substituents in the Napthoquinone moiety weaken the acidity of the compound and this more pronounced when there is a phenyl substituent as in PNQ.

According to Earl (1999) the optimum log P values for oral absorption is 1.8 while that of percutaneous (topical) is 2.6. Among the four compounds under test Buparvaquone has a Log P value of 2.64 making it the most suitable among the four for topical formulation. The rest of the drugs under test have Log P values of 4.21, 6.98 and 9.79 for Parvaquone, NQ and PNQ respectively and as per literature values they are not within the recommended log P values for topical as well as oral formulation.

The difference in log P between buparvaquone and PNQ can be explained by the influence in physico chemical properties by the different R substituents in the two compounds.The tertbutyl cyclohexane substituent in buparvaquone seems to proffer the compound with favourable Log P characteristics for oral formulation (low log P) while phenylbutyl group in PNQ makes highly it more lipophilic (high log P) which is undesirable in oral drug formulation. The aromatic group in the phenylbutyl substituent of PNQ is highly lipophilic hence the high log P.

4.1.3 Distribution coefficient (Log D) measurements

Log D at pH 3.0, 5.0 and 7.4 for the compounds under test were computed using the pKa and log P obtained and the results are summarized in the Table 5 and Figure 11 below;

Table 5. Log D values of the test compounds at pH 3, 5 and 7.4

Drug	Log D		
	pH 3	pH 5	pH 7.4
NQ	6.08	5.83	3.57
PNQ	2.79	2.79	2.77
Parva	4.25	3.17	0.81
Buparv	2.64	2.65	1.67

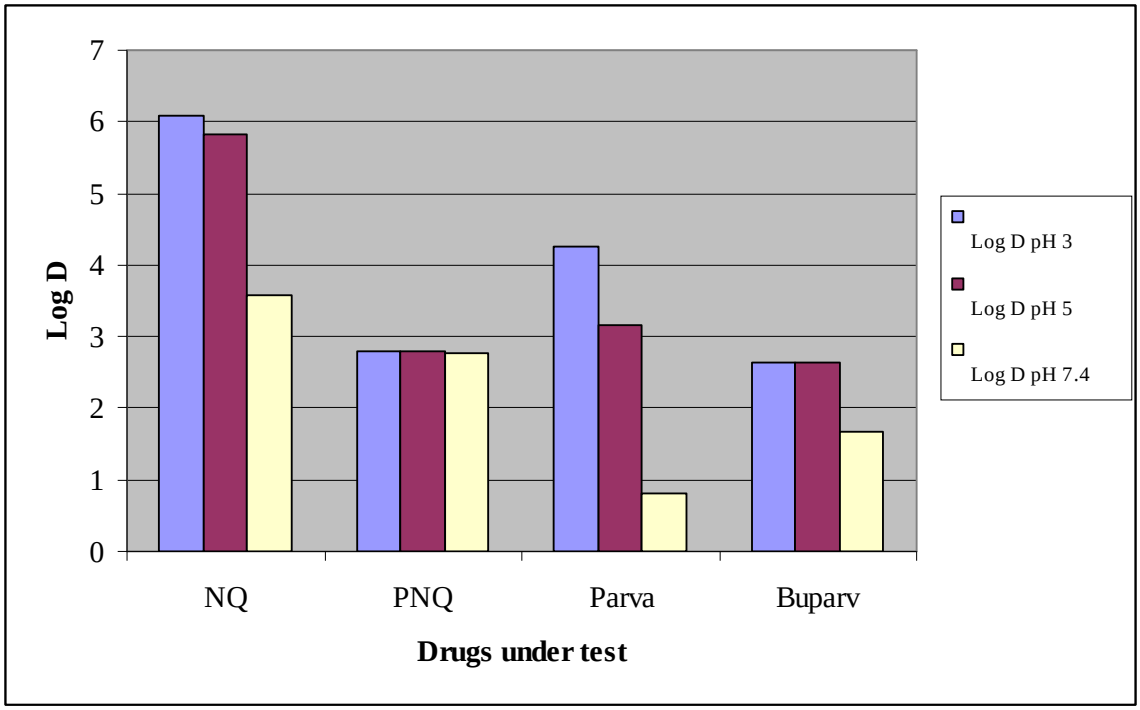


Figure 11. Log D values of drugs under test at varying pH

Key :(i) NQ – 2-Hydroxy -1, 4- Napthoquinone (ii) PNQ - 2-Hydroxy-3-(2-Phenylbutyl)-1, 4-Naphthoquinone (iii) PVQ – Parvaquone (iii) BPVQ - Buparvaquone

The pH gradient in humans begin with a pH of 1 to 3 in the stomach followed by a broader pH range of 5 to 8 in small intestine with the intermediate pH range of around 5 to 6 being found in the duodenum . Colonic absorption in the last segment of the GI tract occurs in an environment with a pH of 7 to 8.

The log D values of Buparvaquone are 2.64, 2.65 and 1.67 at pH 3, 5 and 7.4 respectively while that of PNQ is 2.79 and 2.77 in the same pH range. From the literature (Shalaeva, 2002) these values falls within the optimum range of 1- 3 for orally active drugs. Also at this range they will exhibit low metabolic liabilities. Parvaquone on the other hand has log D values of 4.25, 3.17 and 0.81 at pH 3, 5 and 7.4 respectively. According to literature, compounds with Log D values of 3 – 5 tend to have lower solubility and metabolic liabilities increases while at Log D of 0 - 1 the drug may show a good balance between permeability and solubility.

NQ has log D values of 6.08, 5.83 and 3.57 at pH 3, 5 and 7.4 respectively. According to literature values drugs of log D values above 5 tend to show low solubility and poor oral availability and high metabolic liabilities. From the foregoing the compounds can be ranked from the most suitable candidate for orally active drug to the least and is as follows;

Buparvaquone > PNQ > Parvaquone > NQ

4.2.2 *In vitro* anti-leishmanial assay

Parvequone and related compounds were screened for anti-leishmanial activity against *L. major* and *L. donovani* parasites. The results are summarized in Table 5 -10.

4.2.2.1 Promastigote assay

4.2.2.1.1 MIC assay

The minimum inhibition concentration (MIC) for L. *major* and *L. donovani* promastigotes treated with parvaquone and related compounds are summarized in Table 6 below;

Table 6. MIC values of the compounds under test when using *L. major* and *L. donovani*

Drug	MIC µg/ml	
	L.major	***L.donovani***
NQ	500	500
PNQ	125	125
Parvaquone	1000	1000
Buparvaquone	12.5	12.5
Pentostam	500	500
Amphotericin B	< 12.5	< 12.5

Key :(i) NQ – 2-Hydroxy -1, 4- Napthoquinone (ii) PNQ - 2-Hydroxy-3-(2-Phenylbutyl)-1, 4-Naphthoquinone

The minimum inhibitory concentration (MIC) is the lowest concentration at which parasite motility was observed. For both *L. major* and *L. donovani* the drugs have similar MIC values. The MIC for Buparvaquone at 12.5 µg/ml is the least among the drugs under test and is comparable to that of Amphotericin B (< 12.5 µg/ml) a standard drug for the treatment of Leishmaniasis. The MIC for NQ and PNQ are 500 and 125 µg/ml respectively and while they are much higher compared to Amphotericin B, the results are comparable to that of Pentostam a standard drug which was 500 µg/ml. At 125 µg/ml, the MIC for PNQ is lower than that of pentostam while it is higher than that of Amphotericin B. Parvaquone has the highest MIC among the drugs under test including the standard drugs. Ranking the drugs under test according to MIC yields the following;

Amhotericin B > Buparvaquone > PNQ > NQ, Pentostam > Parvaquone .

The results show that Buparvaquone has as good activity as the standard drug Amphotericin B in promastigote assay and on this basis alone becomes a good candidate for further development and assessment against leishmaniasis parasite.

4.2.2.1.2 MTT assay

The results of MTT assay are summarized below in Table 7, 8 and Figure 12 for *L. major* and *L .donovani* respectively.

Table 7. Optical Density (OD) readings for MTT assay of drugs under test using *L. major.*

Drug	**OD readings at 12.5, 25,50 and 100 µg/ml**			
	12.5	**25**	**50**	**100**
NQ	1.10	1.10	0.98	0.97
PNQ	0.99	0.96	0.92	0.91
Parvaquone	0.99	1.00	0.84	0.84
Buparvaquone	0.66	0.59	0.55	0.57
Pentostam	1.13	0.98	0.67	0.67
Amphotericin-B	0.75	0.75	0.66	0.54
SIM	1.15	1.15	1.15	1.15

Key: (i) NQ – 2-Hydroxy -1, 4- Napthoquinone (ii) PNQ - 2-Hydroxy-3-(2-Phenylbutyl)-1, 4-Naphthoquinone (iii) SIM- Schneider's Complete Insect Media (20% FBS)

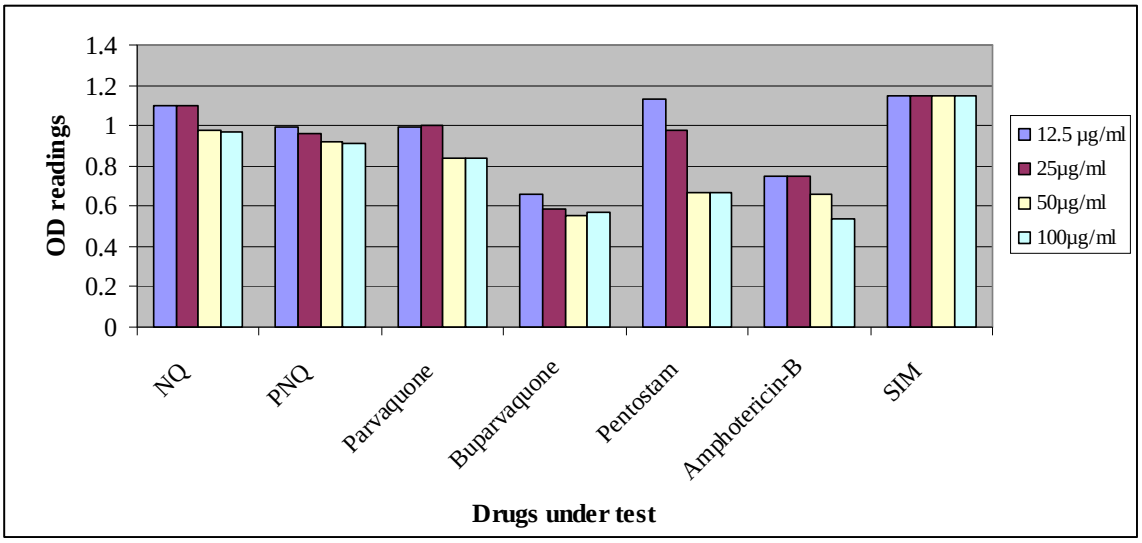


Figure 12. Optical Density (OD) readings vs concentration of the Compounds under test using *L. major*

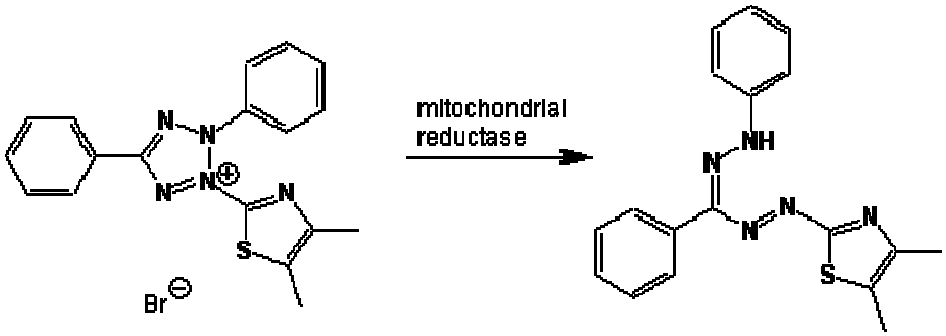
Table 8. Optical Density (OD) readings at varying drug concentration using *L. donovani*

Drug	OD readings at 12.5, 25, 50 & 100 µg/ml			
	12.5	**25**	**50**	**100**
NQ	1.2	0.98	0.94	0.96
PNQ	1.11	1	0.83	0.77
Parvaquone	1.19	1.19	1.13	1.10
Buparvaquone	0.64	0.6	0.59	0.58
Pentostam	1.13	1.01	0.95	0.9
Amphotericin B	0.66	0.58	0.59	0.46
SIM	1.29	1.29	1.29	1.29

Key: (i) NQ – 2-Hydroxy -1,4- Napthoquinone (ii) PNQ - 2- Hydroxy-3-(2-Phenylbutyl)-1,4-Naphthoquinone (iii) SIM – Schneider's complete insect Media (20% FBS)

MTT assay is a laboratory test and a standard colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan, giving a purple color. This mostly happens in mitochondria and as such, it is in large a measure of mitochondrial activity. It can also be used to determine cytotoxicity of potential medicinal agents and other toxic materials. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. A solubilization solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution.

The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption maximum is dependent on the solvent employed. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion is often used as a measure of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death, or changing metabolism of cells, can be deduced through the production of a dose-response curve (Mosman, 1983; Wilson, 2000).



Among the drugs under study, Buparvaquone had the lowest OD readings (0.57) at 100 µg/ml when using *L. major* comparable to the readings of Amphotericin B (0.54) and Pentostam (0.67) which were standard drugs. When using *L. donovani,* Buparvaquone showed the lowest OD reading (0.58) among the test drugs at 100 µg/ml though lower than Amphotericin B (0.46) but higher than Pentostam (0.9) the two standard drugs.

NQ had the highest OD readings for both *L. major* and *L. donovani* at 1.15 and 1.29 respectively among the test drugs but lower than that of SIM which was the positive control.

According to literature cited above, a high OD reading indicates a higher mitochondrial activity therefore a higher number of viable cells and vice versa for lower readings. The results indicate that Buparvaquone had the most potent activity against both *L. major* and *L. donovani* among the test drugs, the activity was comparable to those of Amphotericin B and Pentostam the standard drugs.

NQ on the other hand was the least potent for both species of leishmania even at the highest concentration of 100 µg/ml it showed poor activity. Ranking the order of activity from the most active to the least using the MTT assay gives the following;

Amphotericin B > Buparvaquone > Pentostam > Parvaquone > PNQ > NQ

4.2.2.2 Macrophage assay

Macrophage assay was performed on parvaquone and related compounds and the results are summarized on Table 9 -12 and Figure 13 - 17 below;

Table 9. Infection rate (IR) of macrophages by *L. major* amastigotes at varying drug concentration

Drug	**IR at 50, 25 & 12.5 µg/ml**		
	50	**25**	**12.5**
Pentostam	22	36	56
Amphotericin B	18	26	40
NQ	56	68	82
PNQ	46	54	60
Parvaquone	40	46	50
Buparvaquone	20	36	46

IR in RPMI was 84

Key; (i) L.m. – *Leishmania major*, L.d. – *Leishmania donovani* (ii) NQ – 2-Hydroxy - 1,4 - Napthoquinone (iii) PNQ - 2-Hydroxy-3-(2-Phenylbutyl)-1,4- Naphthoquinone

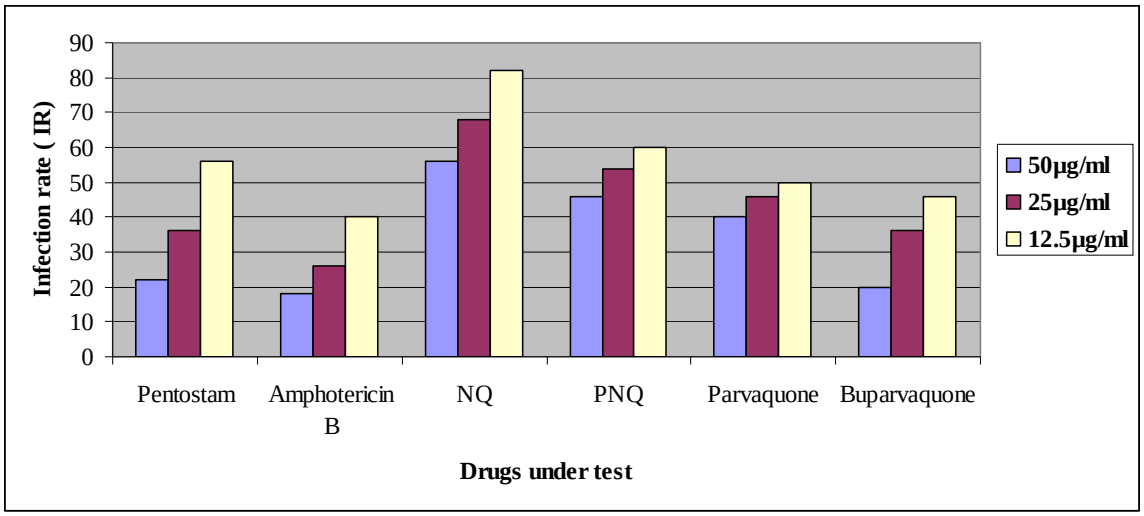


Figure 13. Infection Rate (IR) of macrophages by amastigotes of *L. major* in presence of drugs under test at varying concentration

Table 10. Multiplication index (MI) of *L. major* in presence of test drugs at varying concentration

Drug	**MI at 12.5, 25 and 50 µg/ml**		
	12.5	**25**	**50**
Pentostam	25.35	13.67	8.26
Amp B	24.21	17.09	9.11
NQ	103	59.82	51.28
PNQ	101.42	82.62	71.22
Parvaquone	71.22	42.73	27.06
Buparvaquone	51.28	34.18	14.81

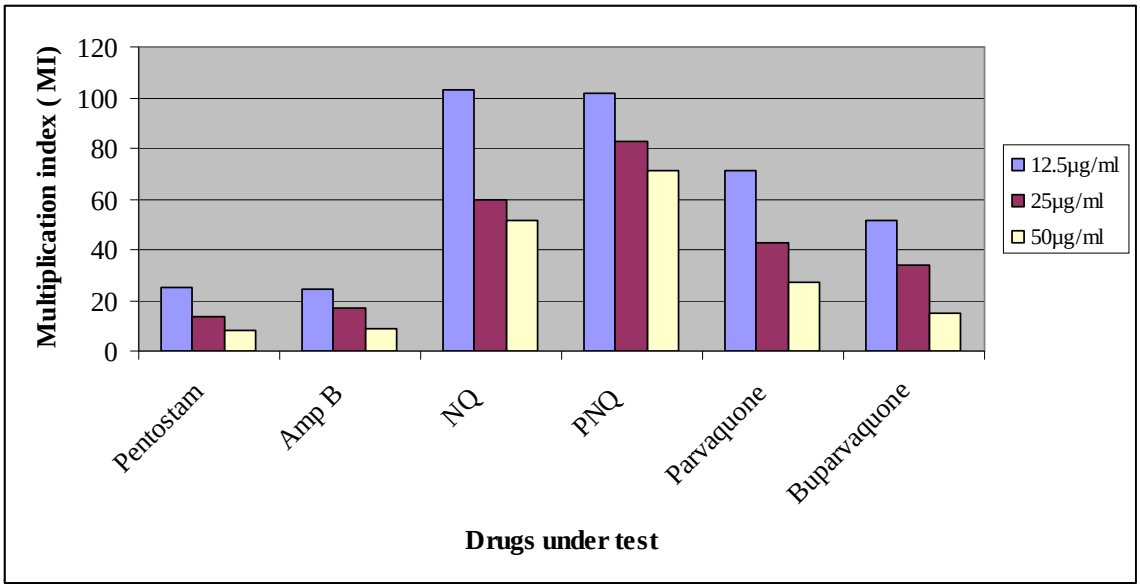


Figure 14. Multiplication Index (MI) of drugs under test at varying concentration using *L. major*

Table 11. Infection rate (IR) of macrophages by amastigotes of *L. donovani* a varying drug concentration

Drug	**IR at 12.5,25,50 µg/ml**		
	12.5	**25**	**50**
Pentostam	58	42	34
Amp –B	36	30	22
NQ	82	56	50
PNQ	68	56	50
Parvaquone	60	52	30
Buparvaquone	50	30	24

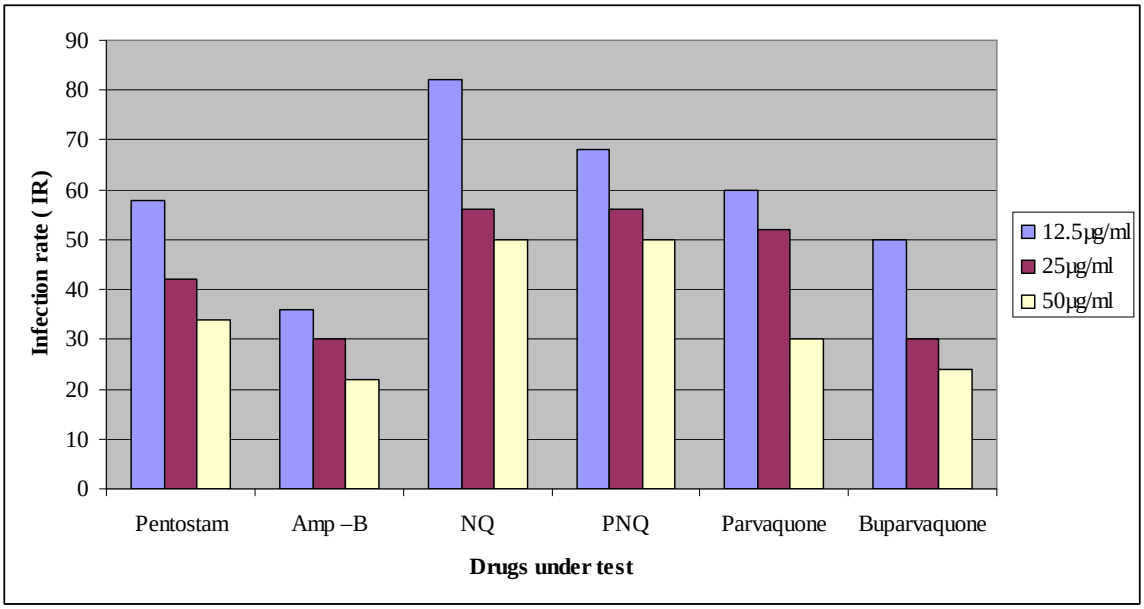


Figure 15. Infection rate (IR) of macrophages by *L. donovani* amastigotes in presence of test drugs at varying concentration

Table 12. Multiplication index (MI) of *L. donovani* at varying drug concentration of test drugs

Drug	MI at 12.5, 25 & 50 µg/ml		
	12.5	**25**	**50**
Pentostam	27.76	14.28	7.27
AMP B	35.04	12.93	8.62
NQ	97.57	61.45	50.13
PNQ	85.98	60.91	56.06
Parvaquone	36.38	35.04	36.38
Buparvaquone	51.48	36.38	14.55

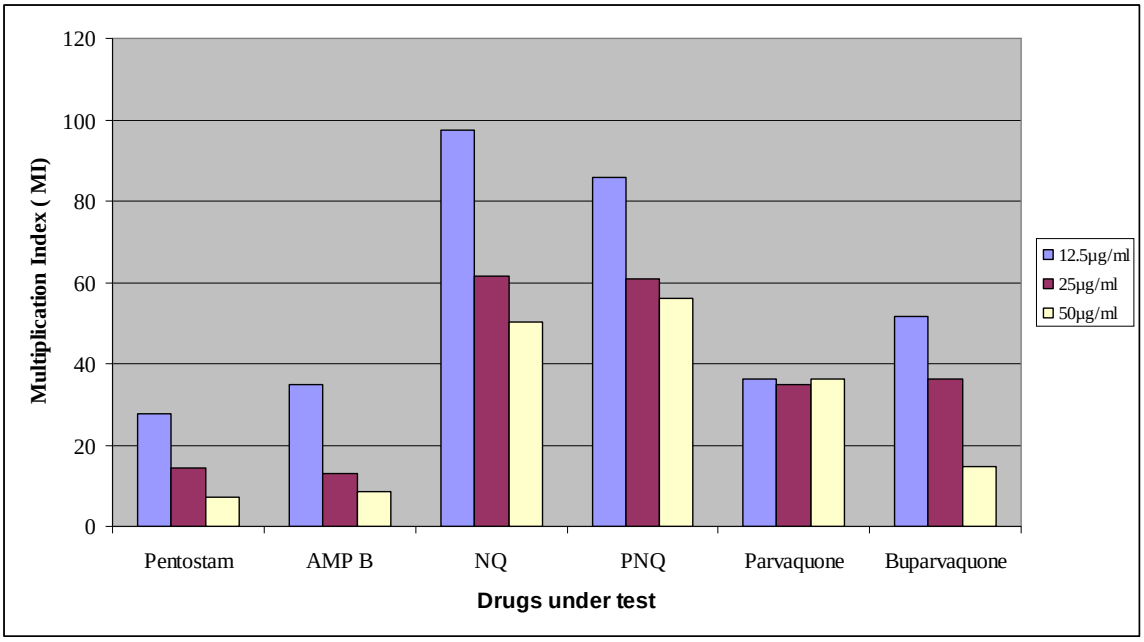


Figure 16. MI versus concentration of drugs under test in presence of *L. donovani*

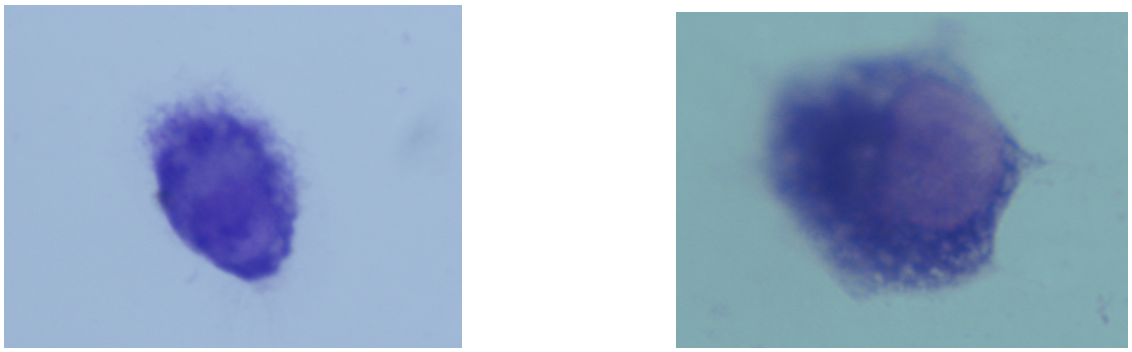


Figure 17. Macrophages infected with *L. donovani* in the negative control group and in presence of 50 µg/ml of Buparvaquone

Infection rate of Pentostam and Amphotericin B (positive controls) at the highest concentration (50 µg/ml) was 22%,18% and 34 %, 22% for *L. major* and *L. donovani*

respectively while that of untreated macrophage cells (with RPMI medium only, negative control) was 84 % and 80% . At highest concentration, pentostam® (positive control) had an MI of 8.26 and 7.27 for *L. major* and *L. donovani* while the untreated cells (negative control) had 100%. At a concentration of 50 µg/ml the lowest infection rate (IR) and multiplication index (MI) among the drugs under test was observed in presence of Buparvaquone at 20%, 14.81 and 24%, 14.55 for *L. major* and *L. donovani* respectively. The highest IR and MI were observed in presence of NQ at 56%, 51.28 and 50%, 50.13 for *L. major* and *L. donovani* respectively.

Ranking the drugs under test starting with lowest IR yields the following;

For *L. major* – Amp B < Buparvaquone < Pentostam < Parvaquone < PNQ < NQ

For *L. donovani* – Amp B < Buparvaquone < Pentostam < Parvaquone < NQ = PNQ

The MI and IR results show that Buparvaquone has good activity against both *L. major* and *L. donovani* comparable to that of Pentostam the standard drug. This was followed by Parvaquone then PNQ and NQ in that order.

4.2.2.2.1 Nitric oxide production determination

A calibration graph of concentration of nitric oxide produced from sodium nitrite was constructed and using the optical readings obtained from ELISA plate reader the graph was used to obtain the concentrations of nitric oxide produced by macrophages treated with different concentrations of parvaquone and related compounds (Table 13) by considering their corresponding optical readings.

Table 13. Nitric oxide (µM) production by macrophages infected with *L. major* and *L. donovani* amastigotes in the presence of test drugs

Drug	***Leishmania* species**	**Nitric oxide production (µM) at 12.5 , 25 and 50 µg/ml of drug**		
		12.5	25	50
NQ	L.m.	0.981	1.095	1.069
	L.d.	1.043	1.043	1.115
PNQ	L.m.	1.084	1.115	1.053
	L.d.	1.095	1.105	1.110
Parvaquone	L.m.	1.022	0.991	1.038
	L.d.	1.053	1.188	1.058
Buparvaquone	L.m.	1.095	0.991	1.012
	L.d.	1.0744	0.981	1.053
Amphotericin B	L.m.	0.991	1.022	1.002
	L.d.	1.04	1.084	1.064
Pentostam	L.m.	0.95	1.022	1.084
	L.d.	0.981	1.022	1.074

L.m- *L. major*, L.d. – *L. donovani* Key :(i)NQ – 2-Hydroxy -1,4- Napthoquinone (ii) PNQ - 2-Hydroxy-3-Phenylbutyl)-1,4- Naphthoquinone (iii)L.m – *L. major* , L.d. – *L. donovani (iv)*The reading for RPMI (-ve control) was 1.105 and 1.043 for L. major and L. donovani respectively.

At the highest concentration (50 µg/ml), Pentostam (positive control) had nitric oxide production of 1.084 µM and 1.074 µM for *L. major* and *L. donovani* respectively while the negative control (RPMI) had 1.105 and 1.043 µM for *L. major* and *L. donovani.* The drugs under test as well as the standard drugs do not show significant production of nitric oxide even when compared to the negative control. It is therefore likely that the drugs under test including the positive control do not kill the leishmania parasite through nitric oxide production.

4.2.2.3 Cytoxicity assay

In order to determine the cytotoxicity of drugs under test an MTT assay was done and results presented in Table 13 and Figure 9 below as % viability of cells.

Table 14. Percent viability of cells at varying drug concentration

Drug	**% viability of cells at concentrations 31.25 - 1000µg/ml**					
	31.25	**62.5**	**125**	**250**	**500**	**1000**
NQ	100	83	77	58	42	36
PNQ	53	44	32	29	28	11
Parvaquone	77	73	73	64	32	26
Bupaparvaquone	87	61	58	36	35	25
Pentostam	80	100	100	100	100	100
Amphotericin B	100	100	100	100	100	100

Key :(i) NQ – 2-Hydroxy -1, 4- Napthoquinone (ii) PNQ - 2-Hydroxy-3-(2-Phenylbutyl)-1,4-Naphthoquinone

From Figure 18 the LC_{50} for the drugs was obtained as concentration at 50% viability for each drug and is shown in Table 15 below along with MIC and Selectivity Index (SI).

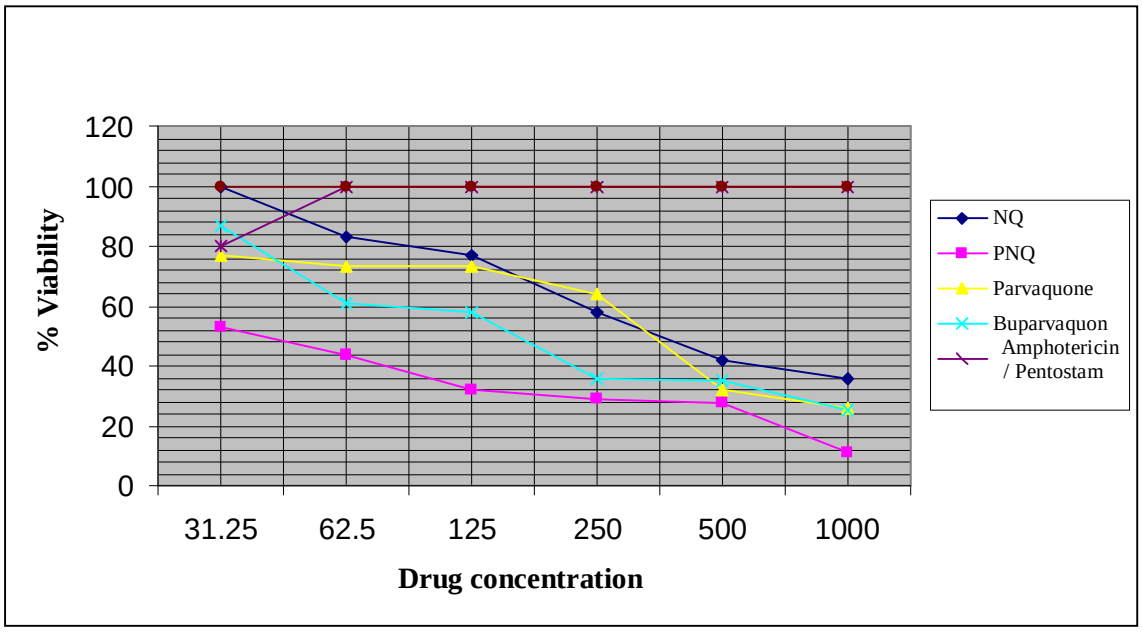


Figure 18. Percent viability of cells versus drug concentration

Table 15. Table to show LC_{50}, MIC and Selectivity Index of the drugs under test

Drug	**LC_{50} (µg/ml)**	**MIC (µg/ml)**	**Selectivity Index (SI) LC_{50}/ MIC**
PNQ	39.06	125	0.31
Buparvaquone	190.00	12.5	15.2
Parvaquone	360.00	1000	0.36
NQ	360.00	500	0.72
Pentostam	> 1,000	500	> 2
Amphotericin B	> 1,000	12.5	>80

LC_{50} is the concentration of the drug in which there is 50% cell viability. The results show that PNQ had the lowest LC_{50} at 39.06 µg/ml while NQ and Parvaquone at 360 µg/ml were the highest among the test drugs. Pentostam and Amphotericin B, the standard drugs (positive controls) had LC_{50} of over 1,000 µg/ml. The SI of Buparvaquone is the highest among the test drugs at 15.2 while PNQ had the lowest with 0.31. The standard drugs had > 2 and > 80 for Pentostam and Amphotericin B respectively. The higher the SI the more favorable for a drug under test as a possible candidate for development as a therapeutic agent. A high SI indicates that the candidate drug is likely to have a wide therapeutic margin, which is the difference between lethal dose (LD_{50}) and therapeutic dose. This also gives an indication of its safety profile at therapeutic doses.

Ranking the test drugs according to SI starting with the highest gives the following;

Amphotericin B > Buparvaquone > Pentostam > NQ > Parvaquone > PNQ

4.3 Summary of results and discussions

The following is a summary of results and discussions;

i) The compounds under test exhibited low aqueous solubility as all were below 65 µg/ml. According to Lipinski (1996), most drugs that are orally active have aqueous solubility greater than 65 µg/ml but when the solubility is 20 µg/ml or less as is the case here, the probability of useful oral activity is very

low. Therefore, the compounds are likely to exhibit low bio - availability due to poor solubility.

ii) The log P value of Burpavaquone was 2.64 which according to literature is optimal for percutaneous (topical) absorption, however it was higher than 1.8 which is the optimal value for oral absorption. The rest of the compounds under test had log P values which were not within the literature values for optimal topical and oral absorption.

iii) The log D values of Buparvaquone and PNQ were within the literature optimum range of 1 - 3 for orally active drugs with low metabolic liabilities (Shalaeva, 2002). The log D values of Parvaquone and NQ were within the range of 3 - 5 and above 5 respectively which according to literature, such compounds will exhibit low solubility, poor oral availability and high metabolic liabilities.

iv) In promastigote assay, Buparvaquone had the least MIC among the test drugs at 12.5 µg/ml comparable to that of Amphotericin B, the standard drug. Parvaquone on the other hand, had the highest MIC twice that of Pentostam the standard drug. The MTT assay further shows that Buparvaquone had the most potent activity against both *L. major* and *L. donovani* promastigotes. The activity was comparable to that of Amphotericin B and Pentostam, the standard drugs. NQ had the least potency among the test drugs.

v) In macrophage assay the lowest infection rate and multiplication index was observed in the presence of Buparvaquone while the highest was observed in

the presence of NQ. This shows that Buparvaquone was the most potent in preventing macrophage infection by leishmania species.

vi) In nitric oxide production determination, the test drugs did not show any significant production of nitric oxide when compared to negative control. It is therefore, unlikely that the mode of action of the test drugs is through nitric oxide production.

vii) In cytotoxicity assay, Buparvaquone had the highest selectivity index (SI) among the test drugs while PNQ had the lowest. A high SI indicates a good safety profile as the drug is likely to have a wide therapeutic margin which is the difference between LD_{50} and therapeutic dose.

CHAPTER 5 : CONCLUSIONS AND RECOMMENDATIONS

5. 1 Conclusions

From the physico-chemical measurements, it is only Buparvaquone among the candidate compounds used in this study that had favourable log P and log D for oral and topical formulation. However, its poor aqueous solubility indicates that there will be challenges in the oral formulation due to poor bioavailability associated with low solubility. In *in vitro a*ssays, Burpavaquone exhibited good activity comparable to those of standard drugs; Amphotericin B and Pentostam. Parvaquone, NQ and PNQ also showed activity but lower than those of standard drugs. The nitric oxide production determination showed that the mode of action for these drugs is not through nitric acid production. Cytotoxicity assay showed that Buparvaquone had the best safety profile among the test drugs while PNQ had the least.

Buparvaquone therefore, presents the most favourable physico chemical parameters as well as good *in vitro* activity for oral and topical activity among the test drugs. However, low oral bio availability due to poor aqueous solubility is likely to present a challenge.

5. 2 Recommendations

i) Parvaquone and Buparvaquone are hydroxy-napthoquinone based drugs that are currently being used in the management of theillerosis (East coast fever) in cattle. Both have demonstrated good *in vitro* activity against leishmania spp in this study particularly Buparvaquone. Theillerosis is a protozoal disease just like Leishmaniasis. This study

therefore, lends credence to the current existing view that other anti-protozoal drugs in the market should be screened for anti-leishmanial activity and those that show good activity should be optimized through Quantitative Structure Activity Relatioships (QSAR) for oral delivery. Developing new anti- leishmanials through existing drugs will guarantee cheaper drugs as costs associated with patents and registration of new drugs will not be there.

ii) Buparvaquone has shown good *in vitro* activity in this study however, poor aqueous solubility presents a challenge for oral formulation. Further studies should be done to improve its solubility without compromising on its activity through introduction of hydrophilic groups such as –COOH, NH_2 and OH in position 8 of the hydroxynapthoquinone moiety. Alternatively by using the OH group in position 2 the compound can be converted in to a phosphate salt with resultant improved solubility.

iii) Buparvaquone demonstrated a good log P value (2.64) for percutaneous (topical) absorption. *In vivo* studies should therefore, be done to determine activity against coetaneous leishmaniasis.

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APPENDICES

APPENDIX 1. Aq. Solubility measurements

Aqueous solubility measurements: UV readings (Absorbance at 650 nm) of the various concentrations of drug solutions under test at pH 4 and 7

i) **Absorbance for Parvaquone at various concentrations at pH 4**

Drug conc. µg/ml	Absorbance at 650nm 1	2	3	Average
0.0015	0.0038	0.0038	0.0038	0.0038
0.003	0.0026	0.0034	0.0038	0.003266667
0.007	0.0001	0.0001	0.0006	0.000266667
0.014	0.0278	0.028	0.028	0.027933333
0.029	0.0132	0.0134	0.0135	0.013366667
0.058	0.0413	0.0413	0.0419	0.0415
0.117	0.0436	0.0516	0.0515	0.0489
0.234	0.0537	0.0537	0.0537	0.0537
0.468	0.051	0.0526	0.0591	0.054233333
0.937	0.001	0.0012	0.0016	0.001266667
1.875	0.0231	0.0231	0.0231	0.0231
3.750	0.0011	0.0018	0.0018	0.001566667
7.50	0.0062	0.0065	0.0068	0.0065
15.00	0.417	0.0393	0.0394	0.165233333

ii) **Absorbance for Parvaquone at various concentrations at pH 7**

Drug conc µg/ml	Absorbance at 650nm 1	2	3	Average
0.0015	0.0065	0.0043	0.0068	0.005866667
0.003	0.0131	0.0128	0.0128	0.0129
0.007	0.0134	0.0129	0.0125	0.012933333
0.014	0.0105	0.1	0.0092	0.0399
0.029	0.0099	0.0092	0.0087	0.009266667
0.058	0.0125	0.0126	0.0123	0.012466667
0.117	0.017	0.0171	0.0176	0.017233333
0.234	0.0586	0.0549	0.0531	0.055533333
0.468	0.0576	0.0555	0.054	0.0557
0.937	0.0835	0.0806	0.0775	0.080533333
1.875	0.0271	0.0253	0.0243	0.025566667
3.75	0.0161	0.0155	0.0151	0.0467
1.50	0.02	0.0188	0.0178	0.018866667
15.00	0.0699	0.0656	0.0624	0.065966667

iii) **Absorbance for BuParvaquone at various concentrations at pH 4**

Drug conc. µg/ml	Absorbance at 650nm			
	1	2	3	Average
0.015	0.0007	0.0009	0.0009	0.000833333
0.003	0.0026	0.0024	0.0024	0.002466667
0.007	0.0024	0.0284	0.0284	0.019733333
0.014	0.0304	0.0348	0.0347	0.0333
0.029	0.0347	0.0348	0.0339	0.034466667
0.058	0.0341	0.0354	0.035	0.034833333
0.117	0.0344	0.0341	0.0334	0.033966667
0.234	0.0341	0.0372	0.036	0.035766667
0.0468	0.0354	0.402	0.0388	0.158733333
0.937	0.0386	0.0403	0.0394	0.039433333
1.875	0.0394	0.0432	0.0433	0.041966667
3.75	0.0432	0.0537	0.0531	0.05
7.50	0.0525	0.0525	0.0524	0.052466667
15.00	0.0613	0.0611	0.0611	0.061166667

iv) Absorbance for Buparvaquone at various concentrations at pH 7

Drug conc µg/ml	Absorbance at 650nm 1	2	3	Average
0.0015	0.0138	0.0125	0.0092	0.011833333
0.003	0.0745	0.074	0.0734	0.073966667
0.007	0.0082	0.0072	0.0063	0.007233333
0.014	0.129	0.012	0.0111	0.0507
0.029	0.0249	0.022	0.0205	0.022466667
0.058	0.105	0.0099	0.0083	0.041066667
0.117	0.0085	0.0088	0.0089	0.008733333
0.234	0.0306	0.028	0.0277	0.028766667
0.468	0.0211	0.0212	0.0212	0.021166667
0.937	0.0343	0.0337	0.0323	0.033433333
1.875	0.0131	0.0132	0.0129	0.013066667
3.75	0.0153	0.0161	0.0149	0.015433333
7.50	0.2487	0.2374	0.2397	0.241933333
15.00	0.1672	0.1667	0.1646	0.166166667

v) **Absorbance for NQ at various concentrations at pH 4**

Drug conc.

µg/ml **Absorbance at 650nm**

	1	2	3	Average
0.0015	0.0043	0.0045	0.0049	0.004566667
0.003	0.0045	0.0045	0.0045	0.0045
0.007	0.0048	0.005	0.005	0.004933333
0.014	0.0059	0.0056	0.0056	0.0057
0.029	0.0052	0.0054	0.0052	0.005266667
0.058	0.0048	0.0046	0.0045	0.004633333
0.117	0.545	0.545	0.5461	0.545366667
0.234	0.0015	0.0012	0.0011	0.001266667
0.468	0.002	0.0016	0.0013	0.001633333
0.937	0.0012	0.0011	0.0013	0.0012
1.875	0.0183	0.0159	0.0148	0.016333333
3.75	0.0228	0.0219	0.021	0.0219
7.50	0.0292	0.0276	0.027	0.027933333
15.00	0.0408	0.0404	0.0404	0.040533333

vi) **Absorbance for NQ at various concentrations at pH 7**

Drug conc	Absorbance at 650nm			
µg/ml	1	2	3	Average
0.0015	0.0015	0.0016	0.0015	0.001533333
0.003	0.0067	0.0072	0.0077	0.0072
0.007	0.0043	0.004	0.0035	0.003933333
0.014	0.0074	0.0079	0.0077	0.007666667
0.029	0.0111	0.011	0.0107	0.010933333
0.058	0.0176	0.0179	0.0181	0.017866667
0.117	0.0398	0.394	0.394	0.275933333
0.234	0.0404	0.0405	0.0408	0.040566667
0.468	0.0515	0.0522	0.0525	0.052066667
0.937	0.0493	0.0488	0.0485	0.048866667
1.875	0.0396	0.0391	0.0387	0.039133333
3.75	0.0422	0.0424	0.0422	0.042266667
7.50	0.0388	0.0377	0.0376	0.038033333
15.00	0.0372	0.0367	0.0367	0.036866667

vii) **Absorbance for PNQ at various concentrations at pH 4**

Drug conc.

µg/ml **Absorbance at 650nm**

	1	2	3	Average
0.0015	0.0023	0.0024	0.0024	0.002366667
0.003	0.001	0.0009	0.0006	0.000833333
0.007	0.0007	0.0005	0.0009	0.0007
0.014	0.0021	0.002	0.002	0.002033333
0.029	0.0194	0.0175	0.0173	0.018066667
0.058	0.026	0.0258	0.0254	0.025733333
0.117	0.0311	0.0305	0.0305	0.0307
0.234	0.0002	0.0002	0.0002	0.0002
0.937	0.0001	0.0002	0.0006	0.0003
1.875	0.0007	0.0006	0.0023	0.0012
3.75	0.002	0.0018	0.0038	0.002533333
7.50	0.004	0.0039	0.072	0.026633333
15.00	0.0708	0.0707	0.0708	0.070766667

viii) **Absorbance for PNQ at various concentrations at pH 7**

Drug conc.	Absorbance at 650nm			
	1	2	3	Average
0.0015	0.0011	0.001	0.001	0.001033333
0.003	0.0002	0	0.0002	0.000133333
0.007	0.005	0.0004	0.0004	0.001933333
0.014	0.0096	0.0101	0.0106	0.0101
0.029	0.1694	0.1691	0.1682	0.1689
0.058	0.0189	0.0189	0.0189	0.0189
0.117	0.0126	0.0123	0.0126	0.0125
0.234	0.0359	0.0364	0.0364	0.036233333
0.468	0.0067	0.0065	0.0062	0.006466667
0.937	0.0087	0.0088	0.0199	0.012466667
1.875	0.0195	0.0284	0.0278	0.025233333
3.75	0.0278	0.0278	0.0082	0.021266667
7.50	0.0068	0.0063	0.1749	0.062666667
15.00	0.1752	0.1753	0.1753	0.175266667

APPENDIX 2. Optical readings of the various drugs under test

MTT assay: Optical Density readings of the various drug concentrations under test

i) **OD readings for NQ at various drug concentrations**

Drug conc. µg/ml	OD readings 1	2	3	Average
31.25	0.535	0.498	0.474	0.5023333
62.5	0.394	0.456	0.399	0.4163333
125	0.492	0.329	0.348	0.3896667
250	0.32	0.287	0.322	0.3096667
500	0.271	0.255	0.201	0.2423333
1000	0.37	0.336	0.334	0.3466667

ii) **OD readings for PNQ at various drug concentrations**

Drug conc. µg/ml	OD readings 1	2	3	Average
31.25	0.24	0.261	0.258	0.253
62.5	0.106	0.068	0.113	0.0956667
125	0.241	0.141	0.175	0.1856667
250	0.239	0.197	0.1175	0.1845
500	0.209	0.153	0.207	0.1896667
1000	0.296	0.284	0.291	0.2903333

iii) OD readings for parvaquone at various drug concentrations

Drug conc.	***OD readings*** ***1***	***2***	***3***	***Average***
31.25	0.341	0.374	0.401	0.372
62.5	0.245	0.163	0.123	0.177
125	0.34	0.515	0.313	0.3893333
250	0.375	0.755	0.456	0.5286667

500	0.149	0.209	0.249	0.2023333
1000	0.357	0.334	0.314	0.335

iv) OD readings of Buparvaquone at various concentrations

Drug conc. µg/ml	**OD readings 1**	**2**	**3**	**Average**
31.25	0.563	0.388	0.348	0.433
62.5	0.242	0.236	0.161	0.213
125	0.342	0.298	0.336	0.3253333
250	0.126	0.113	0.097	0.112
500	0.215	0.292	0.149	0.2186667
1000	0.472	0.176	0.29	0.3126667

v) OD readings of Pentostam at various concentrations

Drug conc. µg/ml	**OD readings 1**	**2**	**3**	**Average**
31.25	0.583	0.568	0.464	0.5383333
62.5	0.533	0.46	0.537	0.51
125	0.546	0.538	0.662	0.582

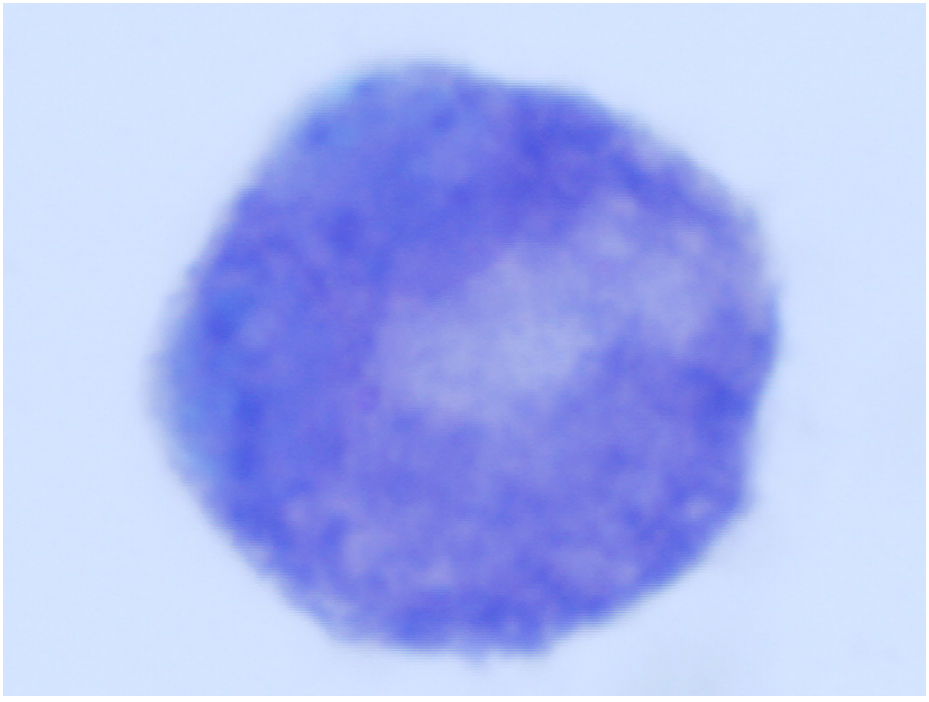
250	0.608	0.701	0.529	0.6126667
500	0.611	0.641	0.619	0.6236667
1000	0.566	0.504	0.742	0.604

vi) **OD readings of Amphotericin B at various concentrations**

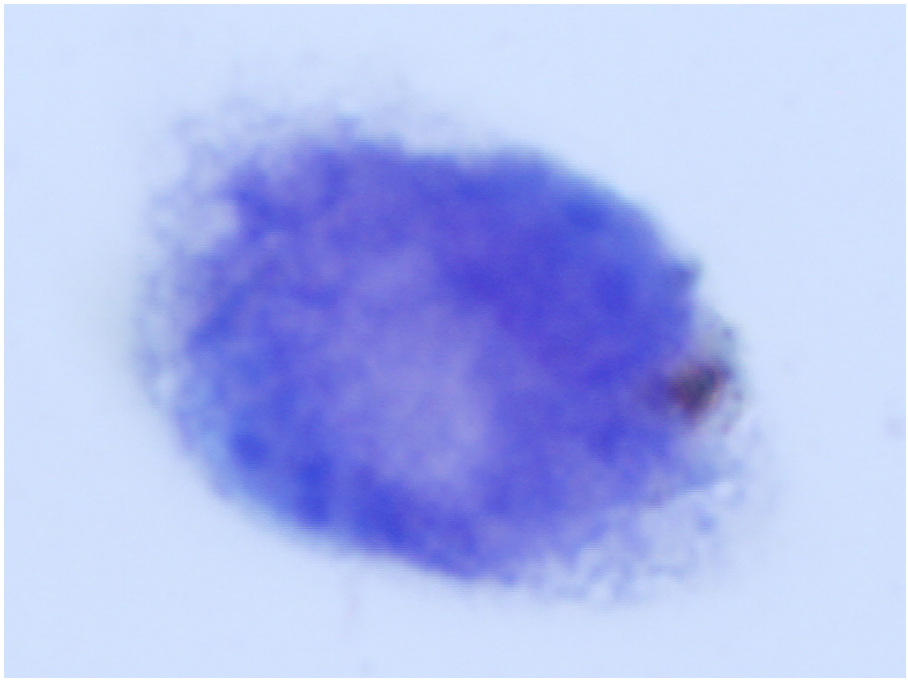
Drug conc. µg/ml	**OD readings 1**	**2**	**3**	**Average**
31.25	0.389	0.414	0.462	0.4216667
62.5	0.55	0.568	0.572	0.5633333
125	0.473	0.497	0.539	0.503
250	0.614	0.534	0.541	0.563
500	0.482	0.486	0.598	0.522
1000	0.579	0.556	0.509	0.548

APPENDIX 3. Macrophages infected with amastigotes in presence of varying concentrations of test drugs

Microscopy: The macrophage pictures below were observed under a compound microscope with x 1, 000 magnification at the KEMRI training centre laboratory.

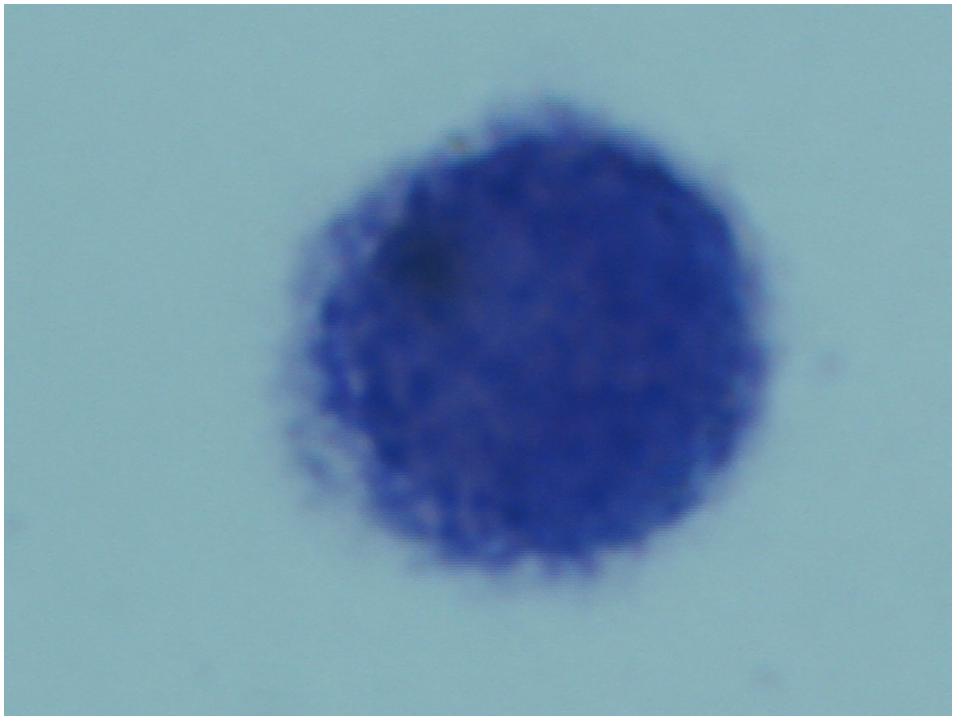


i) A macrophage with amastigotes of *L. donovani* in presence of 50 µg/ml of NQ

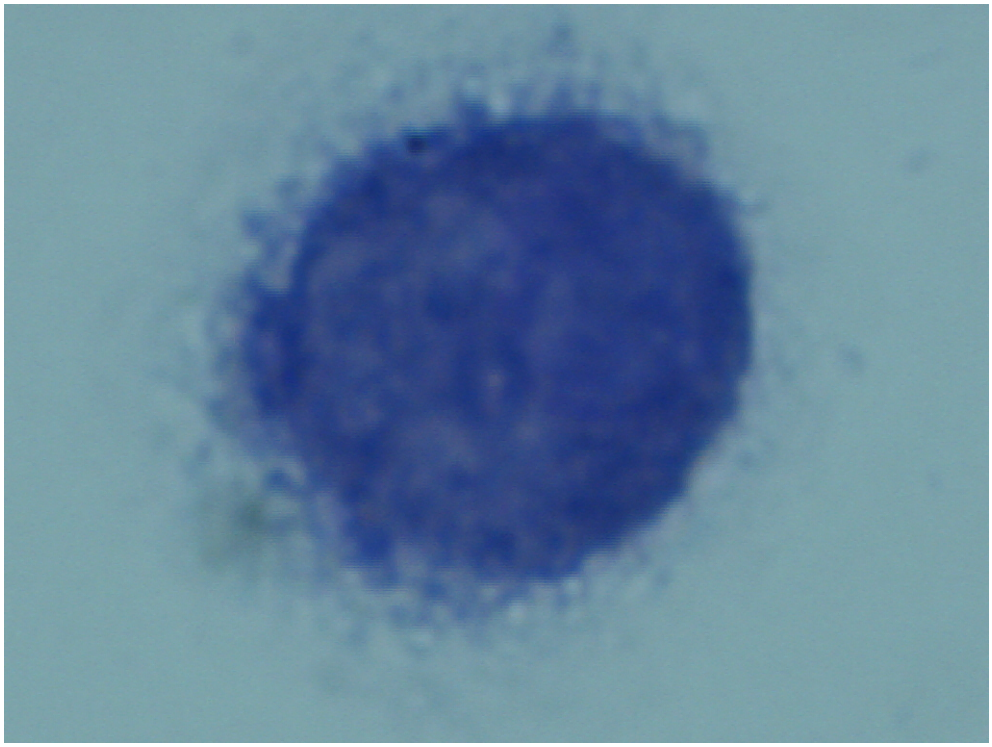


ii) A macrophage with amastigotes of *L. donovani* in presence of 50 µg/ml of

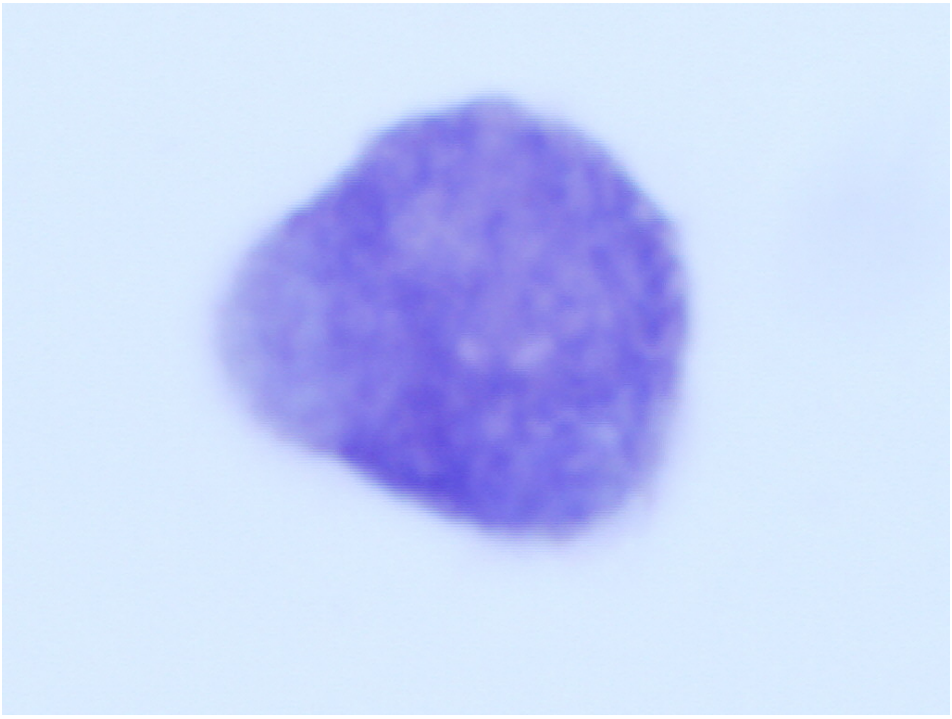
PNQ



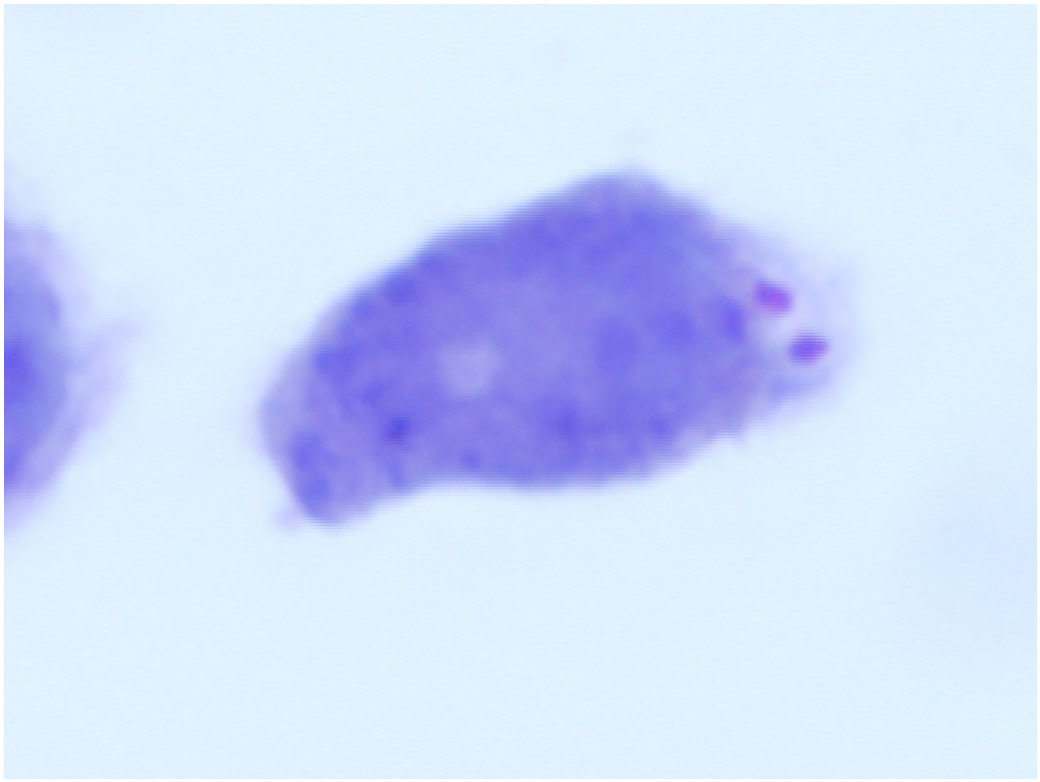
iii) Macrophage infected with amastigotes of *L. donovani* in presence of 50 µg/ml of Parvaquone



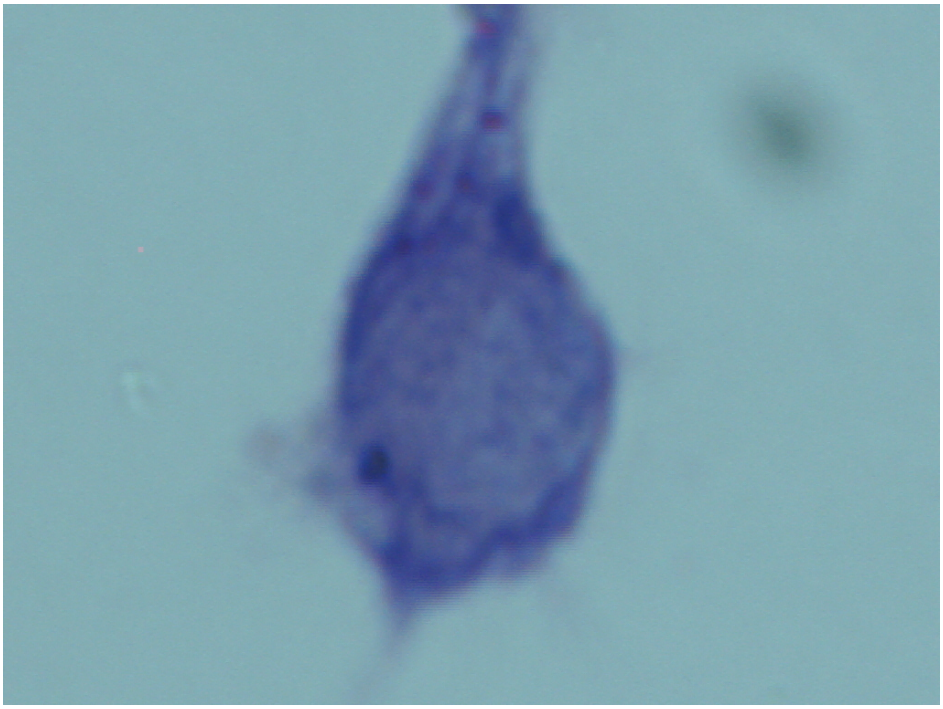
v) A macrophage with *L. donovani* amastigotes in presence 50 µg/ml of Buparvaquone



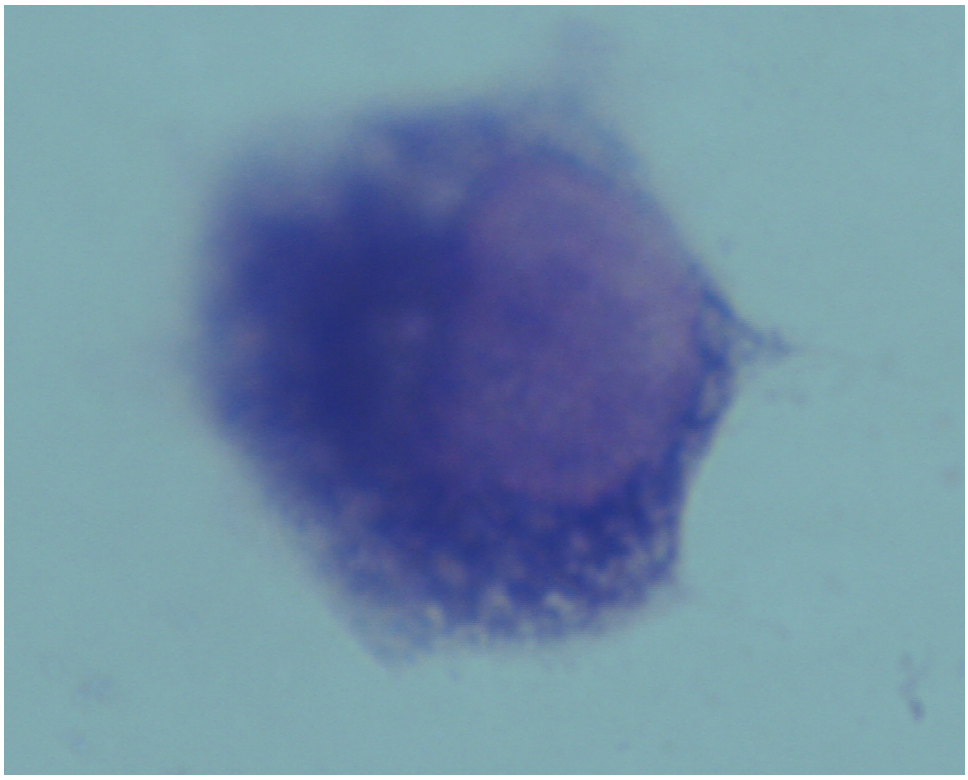
v) Macrophage infected with amastigotes of *L. major* in presence of 50 µg/ml of NQ



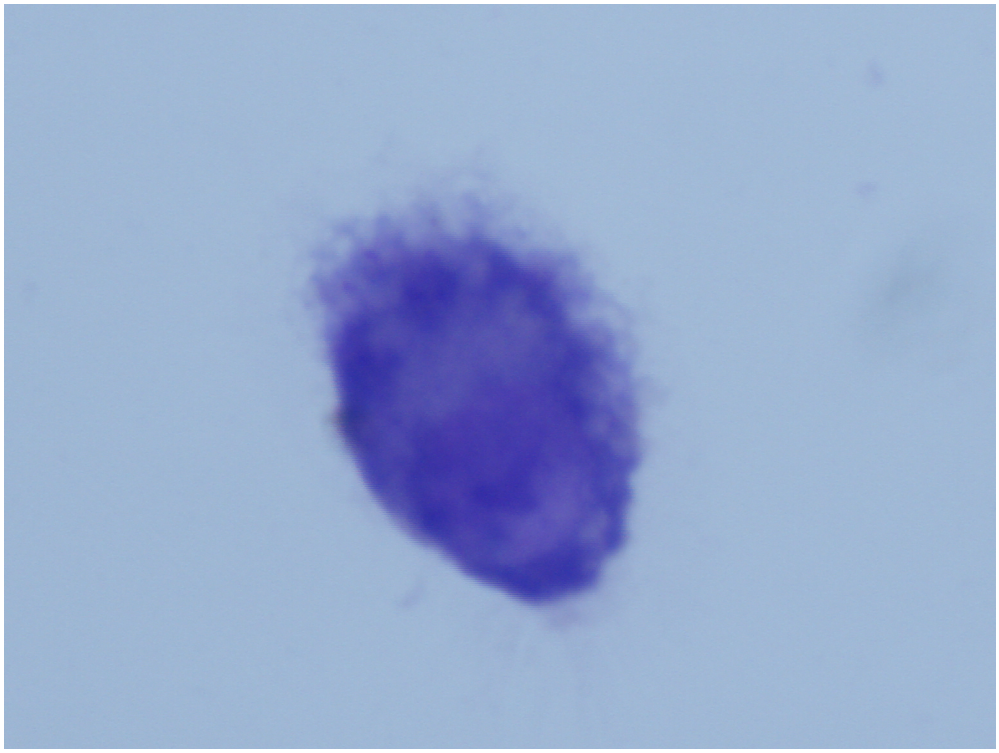
vi) Macrophage infected with amastigotes of *L. major* in presence of 50 µg/ml of PNQ



vii) Macrophage infected with *L. major* amastigotes in presence of 50 µg/ml of Parvaquone

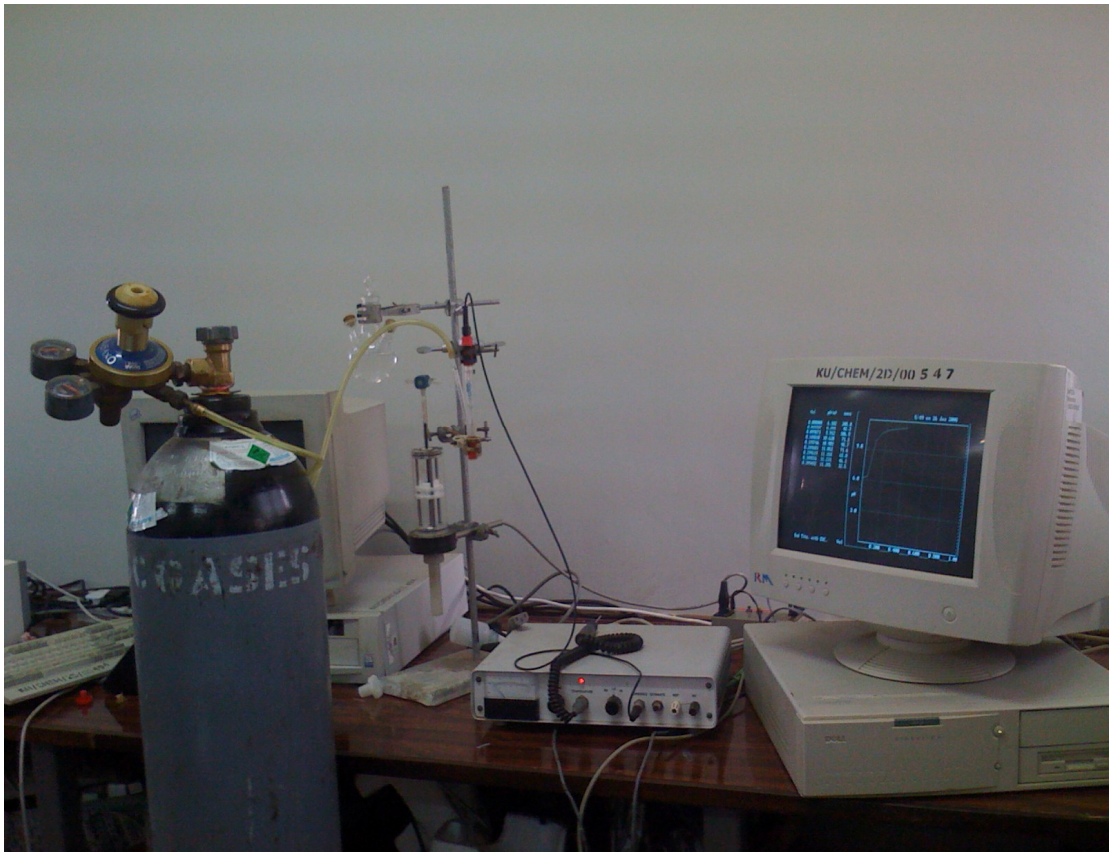


vii) Macrophage infected with *L. major a*mastigotes in presence of 50µg/ml of Buparvaquone



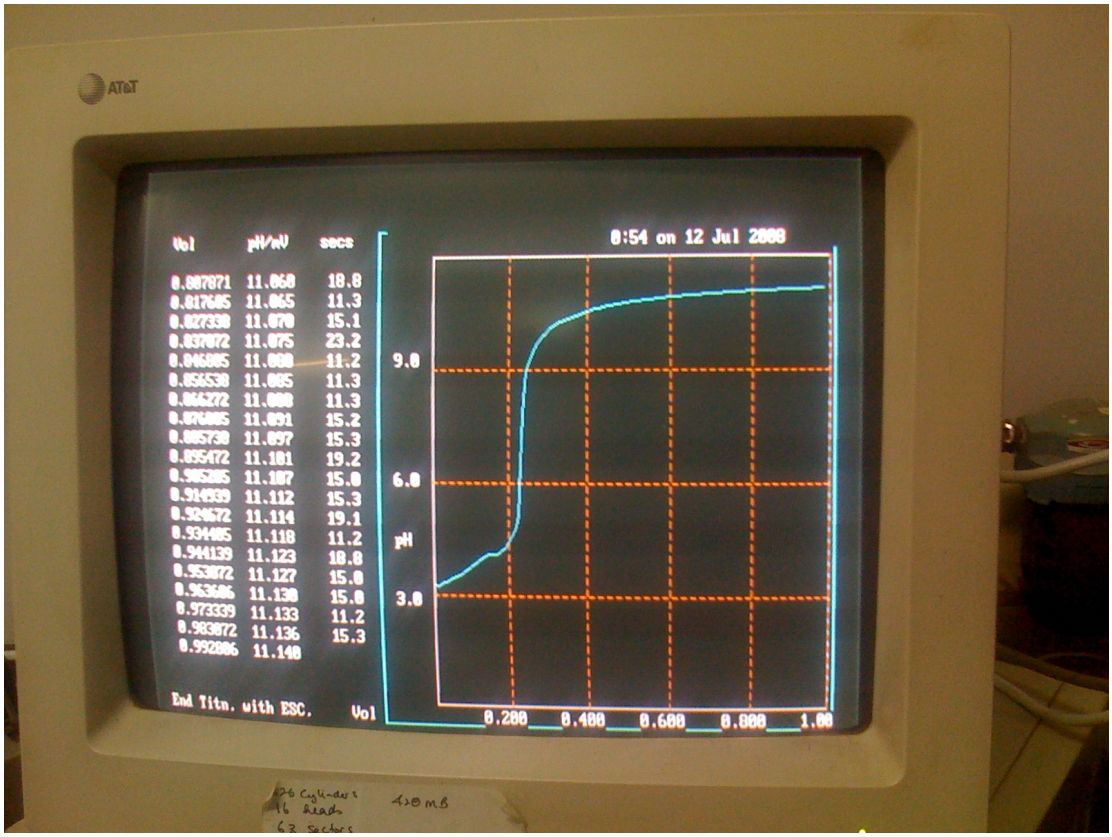
ix) Macrophage in the negative control group infected with amastigotes of *L. donovani*

APPENDIX 4 . Experimental set up for physicochemical measurements

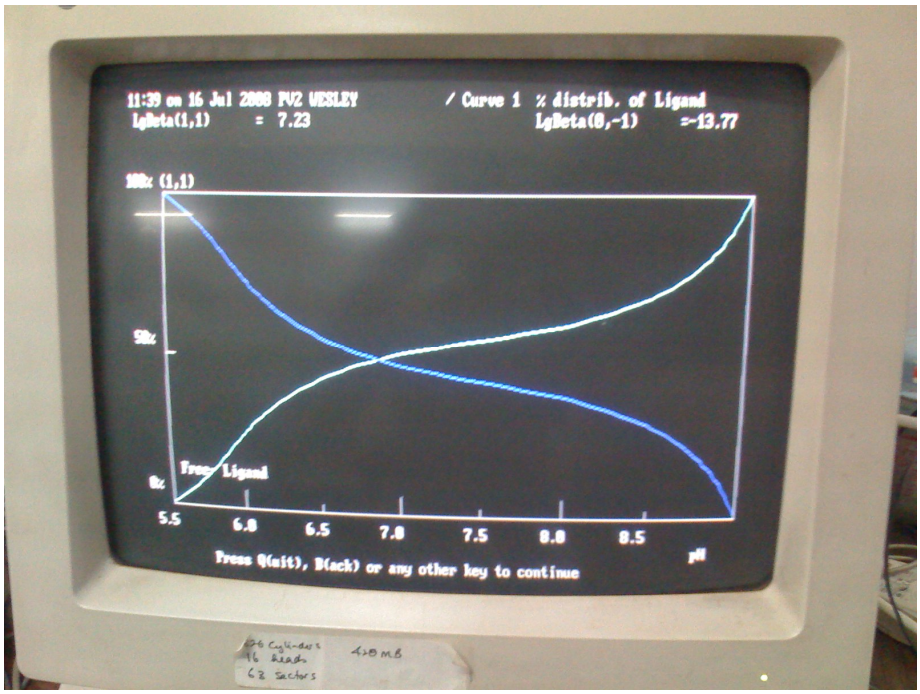


The experimental set up for pKa and log P determination using Molspin pH Auto-titration at the Research Laboratory, Chemistry department, Kenyatta University

APPENDIX 5. A Gran plots obtained using SUPERSQUAD programme



Gran plots obtained using the SUPERSQUAD programme following the MOLSPIN automatic pH titration at the Research Laboratory, Chemistry department, Kenyatta University.


11:39 on 16 Jul 2008 FVZ WESLEY
/ Curve 1 % distrib. of Ligand
LgBeta(1,1) = 7.23
LgBeta(0,-1) =-13.77
100% (1,1)
50%
0%
Free Ligand
5.5
6.0
6.5
7.0
7.5
8.0
8.5
pH
Press Q(uit), B(ack) or any other key to continue