INTRODUCTION

Leishmaniasis, a disease caused by obligate intracellular and kinetoplastid protozoan of the genus *Leishmania*, (Ponte-Sucre, 2013) is an old but largely unknown disease that afflicts the world’s poorest populations (Ngurea *et al.*, 2009). This disease threatens about 350 million people in 88 countries around the world. As many as 12 million people are infected, with an estimated 1 to 2 million new cases arising every year (Dawit *et al.*, 2013). According to disease burden estimates, leishmaniasis ranks third in disease burden in disability-adjusted life years (DALY) caused by neglected tropical diseases and is the second cause of parasite-related deaths after malaria (Bhargava and Singh, 2012).

It is a group of diseases comprising of three clinical entities: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) and muco-cutaneous leishmaniasis (MCL). VL or kala-azar is the severest form of leishmaniasis which is usually fatal if left untreated. It is caused by the viscerotropic species of *Leishmania donovani* in the Indian subcontinent and Eastern Africa, *L. infantum* in the Mediterranean region, and *L. chagasi* in the New World (Nieto *et al.*, 2011). In India, the state of Bihar and adjoining areas of West Bengal, Jharkhand and Uttar Pradesh account for about half of the world’s burden of VL (Sundar and Chatterjee, 2006). All types of leishmaniasis are disseminated by the bite of infected bloodsucking female sandflies that belong to *Phlebotomus* and *Lutzomyia* genera in the Old and New World respectively. There are 500 species of *Phlebotomus* sandflies, of these only 30 species are suspected vectors of this parasite (Herwaldt, 1999; Bora, 1999; Desjeux, 2001; Singh and Sivakumar, 2003; Singh, 2006). In spite of the fact that the dog is the major reservoir for human VL, other animal species may play a significant role in the maintenance of disease. These include chickens, pigs, cattle, and horses kept around the residences in urban areas, which favor proliferation of the vector and therefore transmission of the disease (Diniz *et al.*, 2008). Recently, goats were also considered as the potential animal reservoirs of human VL in India (Singh *et al.*, 2006).
2013). The sandfly ingests the organisms as amastigotes and these transform into promastigotes in its digestive tract and are then injected into susceptible host at the next feed. The promastigotes then infect macrophages and transform into amastigotes (Piscopo and Mallia, 2007).

*Leishmania* infection results in varied clinical and immunopathological manifestations which depends not only on the infecting species of *Leishmania* but also on the ability of parasite to evade the host immune defense mechanisms. For example, VL usually results from infection with either *L. donovani* or *L. infantum* (Mutiso et al., 2013). Systemic symptoms include the gradual onset of fever which may be intermittent or remittent that often rise or fall twice a day, enlarged lymph nodes and liver, fatigue, loss of appetite and thus the weight loss, anorexia, dizziness, cough, diarrhoea, moderate to severe anaemia, pancytopenia, massive splenomegaly, hypergammaglobulinemia and neutropenia, any of which may lead to death without specific chemotherapy (Van Griensven and Diro, 2012). The characteristic feature of kala-azar is darkening or blackening of the skin of hands, feet, face and abdomen. Hair color changes are also sometimes reported. In untreated cases, it also causes severe cachexia, bleeding due to thrombopenia which increases the susceptibility to bacterial infections and then death. Yet, there are many asymptomatic patients which recover spontaneously (Dursun et al., 2009).

Several species have served as animal hosts for VL since the use of humans, for the characterization of a new drug, to understand its immunological aspect and for *in vivo* testing of vaccines, is unethical. Important among them are BALB/c mice and syrian golden hamsters (primary tests), dogs (secondary tests) and monkeys viz. squirrel, vervet and Indian langur monkeys (tertiary tests) (Garg and Dube, 2006). Hamsters are used for histopathological, drug efficacy and vaccine studies. This model mimics several aspects of human disease, such as hepatosplenomegaly, pancytopenia, progressive cachexia, hypergamma globulinemia, and suppression of T-cell proliferative response to parasite antigens. Hamsters reproduce the clinical and pathogenesis of the disease, as seen in humans and dogs. However, the wide use of hamsters is still limited by the lack of available reagents such as antibodies to cell markers and cytokines (de Oliveira et al., 2004; Gupta and Nishi, 2011). Guinea pigs are used
for the studies of delayed type hypersensitivity (DTH) responses. They are the
natural host of *L. enrietti* and have been experimentally infected with other
species of *Leishmania* (Gorezynski, 1983). They have been used as skin test
model to screen potential antigens for use in diagnostic tests for *Leishmania*
(Gorezynski, 1983; Khabiri *et al.*, 2007). But the disease manifestation in the
guinea pig model is not exactly similar to that of humans and thus it has been
superseded by murine model which has been proven to be useful for elucidation
of mechanisms for pathogenesis and immunity in leishmaniasis. In this animal
model, the use of clonal parasite population eliminates the contribution of
genetic diversity of parasites and allows analysis of the host factors which
determine disease manifestations. BALB/c mice are susceptible to *L. donovani*
infections due to the presence of *Scl1a1* gene. Genetically resistant mouse
strains (e.g., CBA) possess a functional *Scl1a1* gene which confers innate
resistance to early *Leishmania* parasite growth. In contrast, susceptible mice
strains (e.g., C57BL/6 and BALB/c) possess a non-functional *Scl1a1* gene and
ever parasite growth in the liver cannot be controlled (Nieto *et al.*, 2011). So,
upon infection, they develop large skin ulcers which expand and metastasize,
leading to death. In mice, the outcome of infection depends on the polarized
activation of one of the two subsets of CD4+ T-cells; Th1 or Th2. Athymic and
*Scid* mice provide a model for treatment of VL in immunosuppressed cases.
*MRL/lpr* is highly susceptible to *L. major* infection. Monkeys are normally the
first experimental animal model to be used in studies on the safety and efficacy
of vaccines and drugs developed in laboratory animals. Many primates have been
experimentally infected with different *Leishmania* species, including the
marmoset, the vervet and the rhesus monkey which are being used to test
potential *Leishmania* vaccines and diagnostic molecules (Kenney *et al.*, 1999;

The incubation period of VL is highly variable; the disease can appear
anytime between ten days to over one year. Even longer incubation periods have
been documented (WHO, 1997). The duration of the disease can be 1-20 weeks,
in endemic areas of Western Sudan the illness usually lasts for about 12-16
weeks with an average of about 6 weeks (Hashim and Elhassan, 1994). VL can
be complicated by serious secondary bacterial infections such as pneumonia,
dysentery and pulmonary tuberculosis, which often contribute to the high fatality rate of VL patients. Other complications though rare include haemolytic anaemia, acute renal damage and severe mucosal haemorrhage (WHO, 1991; Uzair et al., 2004). Early detection of kala-azar is difficult because the clinical and epidemiological findings in various forms of leishmaniasis are non-pathognomonic and these can mimic several other conditions like malaria, tropical splenomegaly syndrome, schistosomiasis, miliary tuberculosis, brucellosis, typhoid fever, bacterial endocarditis, histoplasmosis, malnutrition, lymphoma and leukemia. Hence a laboratory diagnosis is required to confirm the clinical suspicion (Singh and SivaKumar, 2003). Parasitological diagnosis remains the gold standard in the diagnosis of leishmaniasis because of its high specificity. The amastigote forms (called LD bodies) can be seen in tissue smears from lymph nodes, bone marrow or spleen. The sensitivity is highest for splenic aspiration (as high as 98%) but so is the risk of complications such as hemorrhage. Occasionally, the amastigotes have also been demonstrated in liver biopsies (50-85% sensitive), lymph node aspirates and buffy coat smears, particularly in HIV-Leishmania co-infection cases. Splenic aspiration is also associated with serious complications which at times may prove even fatal. Although the specificity is high, the sensitivity of microscopy varies being higher for spleen (93-99%) than for bone marrow (43-86%) or lymph node (53-65%) aspirate (Siddig et al., 1998; Babiker et al., 2007; Srividya et al., 2012).

The biggest problem with conventional smear or culture techniques is low sensitivity particularly in detecting the occult and sub-clinical infections. These techniques are also cumbersome and not suitable for field. Immunodiagnostic techniques may also be used for the diagnosis of VL. They may be based on antigen or antibody detection. The techniques used for antibody detection include gel diffusion, complement fixation, IFA, indirect haemagglutination and countercurrent immunoelectrophoresis. Another test called DAT (Direct Agglutination Test) is a cheap, sensitive and specific test, and its performance is influenced by neither the region nor by the Leishmania species but it is associated with drawbacks like batch to batch variation, instability of antigen, need for incubation and a cumbersome procedure thus, it has not been developed into an affordable commercial test available for use in endemic areas (Maia et
ELISA is also one of the important serodiagnostic tests used in the diagnosis of leishmaniasis. A rapid immunochromatographic test based on a 39 amino acid antigen found in the kinesin region of amastigotes of *Leishmania chagasi*, called rk39 strip test has been developed with sensitivity and specificity estimated at 94% and 95%, respectively. This test currently has an increasing role in VL control programs, since it is easy to perform, cheap, and rapid (10–20 minutes), making it ideal for the use in VL endemic areas. (Van Griensven and Diro, 2012). However the antibody based tests remain positive after drug treatment and cure, therefore cannot readily diagnose relapse. Furthermore, such tests can also detect anti-leishmanial antibodies in asymptomatic individuals living in endemic areas, but with no VL history or subsequent progression to VL (Bhattacharya et al., 2013). Therefore antigen detection is more specific than antibody based immunodiagnostic tests particularly in HIV-VL coinfection, where antibody response is very poor. Two polypeptide fractions of 72-75 kDa and 123 kDa have been detected in the urine of kala-azar patients. The sensitivity of 72-75 kDa fractions was found to be 96% and the specificity was 100%. These antigens were not detectable within three weeks of successful antileishmanial treatment, suggesting that the test has a very good prognostic value (Srivastava et al., 2011). A latex agglutination test (KATEX) for detecting leishmanial antigen in urine of patients with VL has shown sensitivities between 68 and 100% and a specificity of 100% in preliminary tests (Salam et al., 2010). Amplification of the parasite DNA by polymerase chain reaction (PCR) has evolved into one of the most specific and sensitive methods for *Leishmania* detection (Deborggraeve et al., 2008). Diagnosis by PCR is based on the *in vitro* amplification of specific-nucleotide sequences present in the parasite. Several target sequences such as ribosomal RNA (rRNA) genes, the mini-exon derived RNA gene, the kinetoplastid minicircles and repetitive nuclear DNA sequences, etc, have been used in PCR assays (Lachaud et al., 2000; Harris et al., 1998; Pizzuto et al., 2001; Katakura et al., 1998; de Almeida et al., 2011). Real-time PCR assays, Oligo C-Test, Restriction Fragment Length Polymorphism (RFLP), Reverse Dot Blot (RDB), and Reverse Line Blot (RLB) of various genes like kDNA, rDNA, glucose phosphate isomerase (GPI), etc. have also been described for quantification of
*Leishmania* parasites and for differentiation between different species of the parasite (Castilho et al., 2008; Kobetes et al., 2012).

*Leishmania* parasites reside as amastigotes in the macrophages of mammalian hosts therefore, *Leishmania* specific antibodies, disseminated by the immune system of the host cannot approach intracellular phases of the parasite in hepatocytes, splenocytes and bone marrow cells (Garg et al., 2005). Hence protective immune response in experimental leishmaniasis has been shown to be related to the presence of cell mediated immunity and positive delayed type hypersensitivity (DTH) responses to leishmanial antigens in the skin. As for many other intracellular pathogens, it has been assumed that the cellular immunity is involved in the defense against visceral leishmaniasis whereas the humoral arm has little or no role against such pathogens (Ravindran and Ali, 2004). Among T cells, CD4+ T cells are crucial for resistance whereas CD8+ T cells participate more in the memory events of the immune response than as effector cells involved in parasite elimination (Coler and Reed, 2005; Kimutai et al., 2009). CD4+ T helper cells consist of a heterogeneous mixture of two clearly identifiable cellular subtypes Th1 and Th2. There is a correlation between the clinical outcome of infection and the cytokine response profile. Generally, the production of Th2 cytokines leads to severe infection, whereas the production of Th1 cytokines leads to sub-clinical or mild infection (Ghosh and Bandopadhyay, 2003; Kaur et al., 2011). In resistant mouse strains, infection with *L. major* results in the development of a protective Th1 type of immune response with high levels of IFN-γ and resistance to re-infection. By contrast, infection of susceptible mouse strains leads to the development of a Th2 type of immune response characterized by the production of IL-4, IL-6, IL-10 and IL-13, whose role can differ according to the *Leishmania* species (Tacchini-Cottier and Launois, 2008). Studies described that cell mediated immune response is associated with activation of Th1 cells producing IFN-γ, IL-2 and TNF-α. Interferon gamma (IFN-γ) and IL-2 induce nitric oxide-dependent parasite killing by infected macrophages (Ravindran and Ali, 2004; Mougneau et al., 2011). On the other hand, active disease is characterized by a marked humoral response which in turn is associated with expansion of Th2 cells (Reis et al., 2006). These cells secrete anti-inflammatory cytokines IL-4, IL-10 and IL-5.
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During the first week of *L. donovani* infection in BALB/c mice, IL-2 induces IL-10, which suppresses IL-12 production and IL-12 receptor expression on T cells, followed by down-regulation of IFNγ production and parasite proliferation within the macrophage (Salhi et al., 2008). It has been shown that the Th1 cytokine, IFN-γ promotes immunoglobulin switching from IgM to the IgG2a isotype. At the same time, IL-4 triggers switch from IgM to IgG1 and IgE. Indeed, IgG2a and IgG1 kinetics indirectly reflect the Th1/Th2 responses. The relative production of these isotypes can thus be used as a marker for the induction of Th1 and Th2-like immune responses, respectively (Shimizu et al., 2002; Iborra et al., 2005; Lange et al., 2004).

Visceral leishmaniasis (VL) is a chronic parasitic disease and is usually fatal if not treated (Mishra et al., 2013). Pentavalent antimonials, the generic sodium stibogluconate (pentostam) and branded meglumine antimoniate etc, are being used for the treatment of leishmaniasis for decades and still they are the first line drugs of choice where resistance is not reported (Singh et al., 2012). They require injectable administration that can be intravenous (IV), intramuscular (IM) and intralymphatic (IL) (Matoussi et al., 2007) because of poor oral absorption. In the 1980s, dosage studies clarified that a dosage of 20 mg/kg/day rather than 10 mg/kg/day improved the cure rate (Moore and Lockwood, 2010). It causes myocarditis, cardiotoxicity, arthralgia, anorexia, suffocation, fever and may result in death (Cheeran et al., 2010). Moreover in the last few years the emergence of large scale Sbv resistance has been seen in North Bihar, India, where over 60 per cent of previously untreated patients were unresponsive to Sbv rendering the drug useless for routine use (Sundar et al., 2000). The reason for the emergence of resistance is widespread misuse of the drug as Sb(V) is freely available in India, and is easily accessible over the counter (Singh et al., 2012)

Amphotericin B is a polyene antifungal drug widely used to treat systemic fungal infections (Marcondes et al., 2010). In endemic areas of Bihar where antimonials resistance is common, AmB is the drug of choice (Bern et al., 2006). Despite the high efficacy, major limiting factors of Amphotericin B include high fever with rigor and chills, thrombophlebitis and occasional serious toxicities like myocarditis, severe hypokalaemia, renal dysfunction and even death
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(Laniado-Laborin and Cabralis-Vergas, 2009). The predominant toxic effects of amphotericin B deoxycholate have been largely ameliorated with the advent of lipid formulations of amphotericin B. In these formulations, deoxycholate has been replaced by other lipids that mask amphotericin B from susceptible tissues, thus reducing toxicity, and facilitate its preferential uptake by reticuloendothelial cells, thus achieving targeted drug delivery to the parasite resulting in increasing efficacy and reduced toxicity (Sundar and Chatterjee, 2006). Another advantage is that smaller liposomes stay in the blood stream longer than the free drug (Yardley and Croft, 1997). The liposomal formulation (AmBisome) is an approved treatment for VL that besides the reduced toxicity has a better half-life and a high level of efficacy, with 90% cure rate. In experimental VL models, AmBisome has hepatic accumulation, reaches therapeutic levels faster than antimonials and has a longer half-life (Yardley and Croft, 1997; Sundar et al., 2004). The main limitations are its high cost, administration route and lack of stability at high temperature (Cold chain is needed) (Freitas-Junior et al., 2012).

Paromomycin (PM), an aminoglycoside antibiotic, has both antileishmanial and antibacterial activities and has been used in clinical trials for both VL and CL but the limited availability restricts its use in endemic regions (Thakur et al., 2000; Thakur, 2003). Paromomycin administered at 15mg/kg daily for 21 consecutive days has been found to be safe and well tolerated (Sinha et al., 2011). It is considered as the cheapest antileishmanial drug. Ototoxicity and nephrotoxicity are its rare side-effects (Cheeran et al., 2010). Due to its limited use resistance is not yet reported in outpatient treatment but resistance has been reported in vitro in L. donovani and L. tropica (Jhingran et al., 2009; Maarouf et al., 1998).

Pentamidine has been used as a second-line treatment for VL, CL, and DCL for over 40 years. It was initially proven to be useful in Shv resistant kala-azar cases in India but the limiting factors were the expense and above all, the unacceptable toxicity as it causes irreversible insulin dependent diabetes mellitus, rashes, hyperglycemia or hypoglycemia, hypotension, leukocytopenia and thrombocytopenia as well as hepatotoxicity and peripheral neuropathy and death (Rybniker et al., 2010). However, this compound is still valuable for combined therapies (Croft and Coombs, 2003).
Although antimonials (SSG) have long been used as the first-line treatment against VL, it has now been replaced by oral drug miltefosine in the Indian subcontinent due to the emergence of SSG-resistance (Vanaerschot et al., 2011). Hexadecylphosphocholine, miltefosine is a membrane-activating alkylphospholipid simultaneously developed as an anticancerous and antileishmanial agent being recommended as first line drug for childhood VL (Bhattacharya et al., 2004). Miltefosine given for 28 days at the dose of 100-150 mg once a day induced cure rates of approximately 90–95%. Its Phase-IV trials provoked a storm of protection against VL that was followed by phase IV trial which was also found effective especially in VL endemic regions (Sundar and Chatterjee, 2006; Bhattacharya et al., 2007). However its treatment is associated with anorexia, nausea, vomiting (approximately 60%) and diarrhea (approximately 20%). The major limitation of the drug is its long terminal residence and time which could encourage development of clinical resistance (Murray, 2004). Further, its teratogenic and abortifacient nature limits its use in pregnancy (Singh et al., 2012).

It is well known that many antiprotozoal drugs bind to the DNA (Croft and Coombs, 2003). Investigations conducted on kinetoplastid parasites (Trypanosoma cruzi and Leishmania) using cis-DDP or cis-diaminedichloroplatinum (II) or more commonly cisplatin and its analogs have shown that some of these compounds could exert cytotoxic activity (Osuna et al., 1987; Nguewa et al., 2005) suggesting therefore the existence of some parasitic and cancer cell shared regulatory pathways leading to the cis- DDP complexes induced cell death. Cisplatin is a widely used drug in treatment of several types of tumors including testicular and ovarian cancers (Fuertes et al., 2002; Florea and Busselberg, 2011). The chemotherapeutic agent is recognized as a DNA damaging drug. Cisplatin is believed to kill cancer cells by binding to DNA and interfering with its repair mechanism, eventually leading to cell death. cis-DDP is shown to induce alterations in vitro in cell cycle of both promastigotes and amastigotes with the latter being highly sensitive to the drug induced cell death. (Tavares et al., 2007). When tested at a dose of 0.5mg/kg body and 1 mg/kg body wt. in BALB/c mice against visceral leishmaniasis it was found to be protective, but with dose escalation, nephrotoxic effects were observed in the
animals (Kaur et al., 2010). To overcome these effects, the drug was used in combination with various antioxidants which almost diminished all its toxic effects (Sharma et al., 2012).

The development of a vaccine to prevent leishmaniasis has been a goal for almost a century, but currently no such vaccine exists (Kedzierski et al., 2006). First attempts at vaccination, termed leishmanization, were based on the observation that following lesion healing, an individual is resistant to reinfection. This method involved deliberate infection of an individual using live parasites. Nearly 1.2 million people in Iran between 1982 and 1986 received such a live vaccine (Roberts, 2006). The programme in Israel was stopped mainly due to loss of infectivity of the parasite as a result of continuous sub-culturing. In addition, immunosuppression was reported as seen by reduced responsiveness to diphtheria, pertussis and tetanus (DPT) vaccine in children following leishmanization (Khamesipour et al., 2006). The biggest risk with using live vaccines is the problem of reoccurrence of disease in immunosuppressed individuals. The practice of leishmanization has now been largely discontinued based on the grounds of quality control, parasite persistence, emergence of HIV and ethical reasons, among others (Kedzierski, 2011). During the past several decades, extensive efforts have been made to search for an effective Leishmania vaccine. Vaccine formulations including killed, live attenuated parasites, recombinant Leishmania proteins or DNA encoding leishmanial proteins, as well as immunomodulators from sand fly saliva have all been examined (Okwor and Uzonna, 2009). Whole killed Leishmania antigen has been used in both prophylactic and therapeutic vaccines and the latter may have a particular role in drug resistant cases. Theoretically, the killed vaccines from cultured Leishmania parasite should be cost effective in endemic countries. However, their registration was hampered by the lack of standardization. Extensive vaccination trials in Brazil and Ecuador have shown that a cocktail of five killed Leishmania stocks or a single strain of L. amazonensis induces significant protection from natural infection (Bertholet et al., 2005). Phase III trials with a vaccine formulation of killed Leishmania organism mixed with a low concentration of BCG as an adjuvant have also yielded promising results (Gorahava et al., 2013). Preliminary studies using
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autoclaved *L. major* mixed with BCG have been successful in preventing infection with *L. donovani* (Bertholet et al., 2006). Therapeutic vaccines with killed parasite have also demonstrated their good safety profile with encouraging results, despite their poor prophylactic outcomes, in South America and Sudan. Autoclaved and heat-killed antigens of *L. donovani* have also been tested in our laboratory and were found to induce cellular and humoral responses in experimental VL (Nagill et al., 2009). In another study, gamma-irradiated *L. major* elicited substantial protection in mice against challenge with virulent *L. major* (Rivier et al., 1993). Similar results have been obtained with attenuated *Leishmania* derived by long term culture in vitro (Handman et al., 1983; Mitchell et al., 1984, 1985, 1987), selection for temperature sensitivity (Gorezynski, 1985), or chemical mutagenesis (Marchand et al., 1987; Kimsey et al., 1993). Unfortunately, the attenuating defect in these lines has not been defined and they can revert towards virulence (Shankar et al., 1993). There are reports of protective vaccination in mice with genetically modified parasites inducing protection against wild type parasites. The parasite can be modified genetically by gene knock out or by introducing suicidal cassettes (Nagill and Kaur, 2010).

The ideal vaccine is a pan-*Leishmania* vaccine that includes several molecules that are, preferably, conserved among different species and expressed abundantly on the tissue amastigote stage (Coler and Reed, 2005). Antigens thought of as potential vaccine candidates for VL are promastigote surface antigen (PSA) 2 complex, dp72, gp70-72, LACK (*Leishmania* receptor for Activated C Kinase), LeIF (*Leishmania* elongation initiation factor), LCRI (*Leishmania chagasi* recombinant antigen I), Ld23, gp63, FML (Fucose-Mannose Ligand), GRP 78 etc. The most popular of these is the parasite protease, gp63, which is expressed by both promastigote and amastigote stages of most *Leishmania* species (Thiakaki et al., 2006). When used as a recombinant protein expressed in *E. coli*, gp63 either failed to protect mice against *L. major* infection (Handman et al., 1990; Abu-Dayyeh et al., 2010) or offered only partial protection in monkeys (Olobo et al., 1995). In contrast, immunization with the native protein purified from *L. major* led to protection of mice against challenge with either *L. mexicana* or *L. major* (Russell and Alexander, 1988; Rivier et al., 1999). Parasite Surface Antigen 2 (PSA-2) or gp46 is another
vaccine candidate that belongs to a gene family present in all Leishmania species except L. braziliensis (McMahon-Pratt et al., 1992; Basyoni, 2012). Immunization with the native polypeptides derived from promastigotes protected mice against infection (Kedzierski et al., 2004) but vaccination with a recombinant protein derived from either promastigotes or amastigotes protein did not protect mice against infection (Sjolander et al., 1998a). Similarly, DNA vaccination conferred protection in mice when used as either prophylactic (Sjolander et al., 1998b) or therapeutic vaccines (Handman et al., 2000). Another extensively tested antigen is the Leishmania homologue for receptors of activated C kinase (LACK) that is expressed throughout leishmanial life cycle (Mougneau et al., 1995; Sinha et al., 2013). Immunization with LACK appears to promote the expansion of IL-4 secreting T cells skewing the response towards detrimental Th2 responses (Launois et al., 1997). To date, LACK has been found to show protection mainly in the L. major model but it failed to protect against VL (Melby et al., 2001b). Other antigens from different Leishmania species that had been tested in animal models include amastigote cysteine proteases (CP) (Rafati et al., 2005), cysteine proteinase A2, amastigote membrane proteins P4 and P8 (Soong et al., 1995), kinetoplastid membrane protein-11 (KMP-11) (Basu et al., 2005), Leishmania chagasi recombinant antigen (LCR1) (Streit et al., 2000), hydrophilic acylated surface protein B1 (HASPB1) (Stager et al., 2000), leishmanial antigen ORFF (Tewary et al., 2005), acidic ribosomal protein P0 (Iborra et al., 2003), paraflagellar rod protein 2 (PRP-2) (Saravia et al., 2005), NH36, a main component of the fucose-mannose ligand (Aguilar-Be et al., 2005) and proteophosphoglycan (PPG) (Samant et al., 2009). In addition, molecules such as ATP synthase alpha chain, beta-tubulin and heat shock 70-related protein 1 precursor have also been identified as novel vaccine candidates (Bhowmick and Ali, 2008).

A glycoprotein of 78,000 molecular mass (78 kDa), associated with the membrane of parasite is present in equal amounts in both the amastigote and promastigote stages of the organism and is involved in host-parasite interaction (Mukherjee et al., 2002). The glucose-regulated protein 78 (GRP78) of Leishmania donovani is related functionally to members of the 70 kDa stress protein family. HSP70 family members are among the most conserved proteins.
in nature, demonstrating 50–70% amino acid sequence identity in organisms such as bacteria, *Trypanosoma*, yeast and humans. In addition to its classical role as a chaperone, rGRP78 and a GRP78 DNA vaccine protects C57BL/6, C3H/He and BALB/c mice from experimental *L. major* infection (Mukherjee et al., 2002). Moreover, immunization of mice with 78 kDa antigen increased the production of IgG2a titer and reduced spleen weight which correlated with the decrease in parasitemia (Jensen et al., 2001). Moreover, Nagill and Kaur (2010) demonstrated the protective potential of 78kDa+MPL-A in experimental visceral leishmaniasis and found this antigen highly efficacious and effective vaccine candidate against visceral leishmaniasis.

Therapeutic and immunological interventions can affect the outcome of leishmanial infections. Chemotherapy may alleviate leishmaniasis by reducing the parasite load, by changing the leishmanial antigens exposed to the immune system, thus altering cytokine cascade, and by affecting the immune response directly (Ehrenfreund-Kleinman et al., 2005). For the past 70 years, the therapeutic armoury for treatment of visceral leishmaniasis has been extremely limited (Alvar et al., 2006). There are several reasons why consensus has grown over the past few years towards the use of combination regimens in visceral leishmaniasis (Bryceson, 2001; Alvar et al., 2006; Croft et al., 2006; den Boer and Davidson, 2006; Singh et al., 2006; Sundar and Olliaro, 2007). First, combining drugs from different chemical classes could reduce treatment duration or total drug doses, resulting in fewer toxic effects, higher compliance, and lesser burden on the health system (Van Griensven et al., 2010). This could also reduce the overall cost (direct and indirect) and provide a more cost-effective option. Increasing reports of treatment failure with pentavalent antimonials from the Indian subcontinent have raised the issue of acquired drug resistance (Croft et al., 2006; Lira et al., 1999; Sundar, 2001). This concern now extends to miltefosine, because of its long half-life and susceptibility to develop resistance with a single point mutation (Sundar and Murray, 2005; Perez-Victoria et al., 2006; Seifert et al., 2007). Combination therapy might help to delay the emergence of resistance and increase the therapeutic lifespan of the respective drugs, as has been seen for diseases like malaria, tuberculosis, and HIV (Bryceson, 2001; Van Griensven and Diro, 2012). Sundar et al.
(2011) investigated the efficacy and safety of three combinations of three antileishmanial drugs (liposomal AmB, miltefosine, paromomycin) and compared it with the amphotericin B in India, and found combination equally effective as standard monotherapy but with fewer side effects and shorter course of administration (Jain and Jain, 2013).

So, an ancillary therapeutic measure that might enhance the efficacy of these antileishmanials or reduce the resulting toxicity would be valuable. The immune status of leishmaniasis patients has long been known to affect drug efficacy. This has to be of particular importance in relation to pentavalent antimony treatment of DCL (Haldar et al., 2011) and coinfections with HIV in the visceral form (Berhe et al., 1999; Desjeux and Alvar, 2003), where there is an absence of a specific T-cell-mediated immune response and mutual exacerbation of infection. The basis for this lack of activity of pentavalent antimonials has been explored in immunodeficient mouse models for which the effects are probably due to deficiencies of both Th1-cell-mediated and macrophage responses (Murray et al., 1989; Haldar et al., 2011). Immunochemotherapy has also been used with various combinations of drugs and vaccines mostly in case of cutaneous leishmaniasis. Some of them are sodium stibogluconate with poly ICLC (Polyinosinic-polycytidilic acid) plus arginine (Bhakuni et al., 1996), killed Leishmania promastigotes with antimonials (Machado-pinto et al., 2002), n-methyl meglumine antimoniate with recombinant Leish-110f plus MPL-SE vaccine (Miret et al., 2008), and alum precipitated autoclaved Leishmania promastigote (ALUM/ALM) plus BCG with sodium stibogluconate (Musa et al., 2008).

Thus immunotherapy with the help of cytokines, vaccines or other immunomodulators could be an effective addition to chemotherapy for leishmaniasis. Keeping this in mind, the present study was designed to assess the in vivo efficacy of immunochemotherapy with the use of first and second generation vaccines along with sodium stibogluconate and a novel drug cisplatin and to compare them with chemotherapy and immunotherapy alone.