CHAPTER 1

INTRODUCTION
**Introduction:** Leishmaniasis, a complex vector borne parasitic disease is caused by obligate intra-macrophage protozoan parasites of the genus *Leishmania*, a member of the order Kinetoplastida. Endemic in 80 countries, the disease is a major global health problem. The World Health Organization (WHO) estimates that leishmaniasis is affecting 12 million people and threatening 350 million more with an estimated 1.5-2.0 million new cases and 80,000 deaths every year (Verma and Dey, 2004). Leishmaniasis has emerged as the third most prevalent parasite-borne disease worldwide after malaria and filariasis. The disease is manifested in three forms, namely visceral, mucocutaneous or cutaneous; of which the severest is the visceral affliction caused mainly by *Leishmania donovani* and *Leishmania infantum* with an estimated 500,000 new cases reported annually (Desjeux, 2004). Over 90% of these visceral cases occur in Brazil, Bangladesh, India, Nepal and Sudan, although these numbers are likely to be an underestimation since majority of the cases are not even reported in these and many of the other 88 endemic countries. Cutaneous leishmaniasis (CL), caused by *Leishmania tropica* and *Leishmania major*, is the commonest presentation representing up to 75% of all new cases, wherein lesions appear on the face, arms and legs. Mucocutaneous leishmaniasis (MCL) caused by *L. braziliensis* and *L. amazonensis* is manifested by nasal obstruction and bleeding and generation of painful mucosal lesions. Visceral leishmaniasis (VL) caused by *L. donovani* and *L. infantum* (named *Leishmania chagasi* in the Américas) is manifested by persistent low-grade fever, hepatosplenomegaly, cachexia, pancytopenia and hypergammaglobulinemia. The parasites reside primarily within macrophages of the liver, spleen, and bone marrow, and the course of the disease indicates an underlying defect in immune defense mechanism of the host. It is the second largest parasitic killer after malaria and almost always results in the death of the host, if left untreated (Desjeux, 2004). *Leishmania* genus exists in two forms that develop in two different hosts. Flagellated extracellular infective form, the promastigotes live inside the digestive tract of female sand fly vector while the non-flagellated intracellular amastigotes reside within the host macrophages (Desjeux and Alvar, 2003). Leishmaniasis is one of the opportunistic infections that attack human immunodeficiency virus (HIV) infected individuals, and in the recent years, the coexistence of HIV and *Leishmania* species causing visceral disease has resulted in several thousand cases of dually infected individuals. Leishmaniasis patients are highly susceptible
to HIV infection, and leishmaniasis accelerates the onset of AIDS by cumulative
immunosuppression and by stimulation of the replication of the virus in HIV-infected
patients (Carvalho et al., 2000).

BALB/c mice are highly susceptible to infection with the parasite *Leishmania* sp. This
susceptibility has been attributed, in part, to the expansion of Th2 cells and production of
their cytokines, interleukin (IL)-4 and IL-10, and down regulation of Type 1 helper (Th1)
cytokine, interferon gamma (IFN-γ) (Heinzel et al., 1991). The inability of susceptible
hosts to mount the immune response necessary to activate macrophages and destroy the
parasites can be due to the parasite-specific proteins that are able to modulate the immune
system (Abolhassani et al., 2004). Resolution of infection is associated with the induction
of macrophage nitric oxide (NO) (dependent upon inducible nitric oxide synthase (iNOS)),
IFN-γ, reactive oxygen species (ROS), specific CD4+ Th1 and CD8+ lymphocyte
responses (Liese et al., 2008). *Leishmania* species employ distinct mechanisms to elude
effector arms of the immune system, including inhibition of NO- and ROS-mediated
macrophage killing and phagolysosomal fusion, inhibition of cell-mediated immunity via
blocking of antigen presentation and cytokine production, and recruitment of regulatory T
cells or other IL-10 producing cells (Nylen et al., 2007).

Even during chemotherapy, the cure of leishmaniasis, appears to be dependent upon the
development of an effective immune response that activates macrophages to produce toxic
nitrogen and oxygen metabolites to kill the intracellular amastigotes (Alvar et al., 1997,
Murray et al., 1989). This process is suppressed by the infection itself, which down
regulates the requisite signaling between macrophages and T cells, such as the production
of IL-12 and the presentation of major histocompatibility complex (MHC) and co
stimulatory molecules at the macrophage surface (Murray et al., 2005). The treatment of
VL is difficult because of intra-macrophagic location of amastigotes. Victims of this illness
present an immune deficiency and are not able to eliminate the parasites through a natural
mechanism of defense (Myler and Fasel, 2008). The problem of VL has been worsened
due to parallel infection in acquired immuno deficiency syndrome (AIDS) patients
(Nazmul, 2007).
For the last 70 years, sodium antimony gluconate (SAG) has been the mainstay of antileishmanial therapy. However, its use is associated with hazardous iatrogenic effects like severe cardiotoxicity accompanied by congestive heart failure, ventricular tachyarrhythmia and ventricular fibrillation that could be fatal (Sundar et al., 1998). Further limitations are its variable efficacy (Guerin et al., 2002) and of greater concern is the steady erosion in its efficacy and development of resistance to pentavalent antimonials in patients with VL especially in North Bihar, India, that has further increased the problems of treatment (Sundar et al., 2003). Chemotherapy of VL also includes Amphotericin B and its lipid formulations but limitations are their parenteral administration, toxicity and high cost (Guerin et al., 2002). Miltefosine, originally developed as an anticancer drug is the first orally effective antileishmanial drug but is costly and teratogenic, and requires monitoring for gastrointestinal toxicity, hepatic toxicity, and nephrotoxicity (Sundar et al., 2007). Due to long treatment durations, development of resistance against miltefosine is a major concern (Sundar et al., 2007). Hence in the absence of vaccines, the search of novel, effective and safe therapeutic compounds against leishmaniasis has become a priority (Vennerstom et al., 1990).

In an ongoing search for better and cheaper leishmanicidal agents, plant-derived secondary metabolites are an attractive option (Akendengue et al., 1999; Kayser et al., 2003). According to world health organization (WHO), approximately 80% of the world inhabitants rely on traditional medicines for their health care (Newman and Cragg, 2007). The harmonization of traditional and modern medicine is one of the principle goals in the progressive playground of pharmacognosy (WHO, 2000). India is rich in traditional medicinal plant species, providing opportunities to explore and exploit this resource for various diseases and metabolic disorders. The role of natural products as a source for remedies has been recognized since ancient times (Cragg et al., 1997). Plants have been an integral part of the ancient culture of India, China and Egypt as medicine, and their importance dates back even to the Neanderthal period (Solecki, 1975). In fact, so strong is the reputation of plants as healers that the word “drug” has been found to have its origins from the latin “droughe” meaning “dry herb”. The use of medicinal plants and studies on the chemistry and pharmacology of natural products have grown considerably in the second half of the 20th century (Albuquerque et al., 2007). Recent analysis showed that for
the period, 1989 to 1995 over 60% of the approved drugs developed in areas of cancer and infectious diseases were of natural origin. Compounds derived from natural sources have pharmacological activities with minimum side effects and may be used for drug design and discovery process. The antimicrobial activity of plant oils and extracts has been employed in many industrial applications like food preservation, pharmaceuticals, alternative medicine, drugs, perfumery and natural therapies (Reynolds, 1996). Plant products as antimicrobial agents have aroused interest because of the challenge from antibiotic resistant microorganisms (Essawi and Srour, 2000).

The use of plant products as immune-stimulants has a traditional history. Herbal drugs are believed to enhance the natural resistance of the body against infections and their immunomodulatory activities have been reported in numerous plants (Geoffrey et al., 1994). The concept of immunomodulation relates to nonspecific activation of the function and efficiency of macrophages, granulocytes, complement, natural killer cells and lymphocytes and also to the production of various effector molecules generated by activated cells. It is expected that these nonspecific effects may augment protection against different pathogens through preferential induction of Th1-like immune response and constitute an alternative to conventional chemotherapy (Vigila & Baskaran, 2008).

In this regard, plants are imperative sources of diverse types of bioactive organic compounds, including the complex group of essential oil-related compounds. Essential-oil constituents contribute to beneficial or adverse effects based on their characteristic biological activity. Essential oils are known to possess antibacterial and antifungal properties against human pathogenic microorganisms (Fyfe et al., 1997). Essential oils obtained from Achillea millefolium, Ocimum basilicum (Santoro et al., 2007) and Artemisia spp. exhibit toxic effects against trypanosomes. A plethora of studies indicate promising leishmanicidal effect of essential oils from various plants including Croton cajucara (Rosa et al., 2003), Ocimum gratissimum (Ueda-Nakamura et al., 2006) and Artemisia herba-alba (Mohamed et al., 2010), Eugenia uniflora (Rodrigues et al., 2013) and Origanum vulgare (Sanchez-Suarez et al., 2013). Aloe vera exudates are known to cause in vitro and in vivo antileishmanial efficacy in experimental VL (Dutta et al., 2008).
Fractions obtained from crude extracts of leaves of *cajazeira spp* and *Musa spp* tree have shown leishmanicidal effect on promastigotes and amastigotes of *Leishmania donovani in vitro* (Marina *et al.*, 2011). Experimentally it has been observed that herbal immunomodulators from *Mangifera indica, Ocimum sanctum, Phyllanthus emblica* and *Withania somnifera* are very helpful in boosting the immune system (Singh *et al.*, 2007). Ethanolic extract of *Nyctanthes arbor-tristis* potentially stimulates immune system and affects both humoral immunity as well as cell-mediated immunity as shown by its effect in the indirect hemagglutination test and serum immunoglobulin levels (Kannan *et al.*, 2007). *Picrorrhiza kurroa* has been reported by Atal *et al.*, (1986) to be promising immunomodulatory agent. It has been used to enhance antibody and delayed type hypersensitivity (DTH) response to sheep red blood cells (RBC) in mice. Picroliv, an iridoid glycoside derived from this plant was found to significantly protect golden hamsters against challenge with *Leishmania donovani* promastigotes (Puri *et al.*, 1992).

*Artemisia annua* (Asteraceae), a well-known traditional medicinal plant, has been extensively used as antimalarial and anticancer agent. Essential oil from *A. annua* has been reported to exhibit antifungal, antibacterial (Juteau *et al.*, 2002) and acaricidal activity (Pirali-Kheirabadi and Teixeira da Silva 2011). Recently the in vitro and in vivo efficacy of artemisinin (one of the constituents of *A. annua, A. indica* and *A. dracunculus*) against hepatocellular carcinoma (Hou *et al.*, 2008) and against experimental VL has been studied (Sen *et al.*, 2010). The antileishmanial activity of the ethanolic extracts of the leaves of *A. indica* was investigated in exponential phase of promastigotes from six strains and IC$_{50}$ (the concentration that inhibit 50% cell growth) was found to range from 210 μg ml$^{-1}$ to 580 μg ml$^{-1}$ (Ganguly *et al.*, 2006). The flavonoids from *A. annua* were shown to be antioxidant and their potential synergism with artemisinin against malaria and cancer has also been studied (Ferreira *et al.*, 2010). In the present study, we have investigated the leishmanicidal activity of *A. annua* (leaves and seeds) fractions and essential oil from fresh leaves on both promastigote and amastigote forms of *L. donovani in vitro and in vivo* as well their immunomodulatory effects to assess whether the fractions could differentially affect the immune response to *Leishmania* parasites.
The work embodied in this thesis represents the broad spectrum *in vitro* and *in vivo* antileishmanial efficacy and immunomodulatory activity of crude fractions and essential oil of *Artemisia annua* in experimental VL. The *in vivo* study was evaluated using BALB/c mice model.

**AIMS AND OBJECTIVES OF THE STUDY**

In this study, we proposed to explore the leishmanicidal and immunomodulatory activities of *Artemisia annua* (leaves and seeds) extracts, their bioactive molecules and essential oil against experimental VL. The objective has been achieved as follows:

1. Screening of the extracts from leaves and seeds of *A. annua* against *L. donovani* *in vitro*
2. *In vitro* evaluation of the mode of death of promastigotes by the bioactive extracts.
3. Evaluation of *in vivo* antileishmanial efficacy of the bioactive extracts in *L. donovani* infected BALB/c mice using amphotericin B as positive control.
4. Elucidation of the mechanisms of protective immunity (cell-mediated and humoral) elicited by the bioactive extracts.
5. Study of the adverse toxicity of the bioactive extracts *in vitro* as well as *in vivo*. 