SUMMARY AND CONCLUSIONS

Robust treatment is the major strategy to cure visceral leishmaniasis. There are number of drugs available in the market for the treatment of this hyper-endemic disease. However the cases of VL are increasing at an alarming rate worldwide. The main reasons for the re-emergence and spread of disease are the treatment failures due to the evolution of resistant parasites and asymptomatic and PKDL patients. For instance SSG has been abandoned in many regions of India due to critical issues like growth of resistance and toxic-effects of drug. Thus in parallel to the discovery of safe antileishmanial agent it is essential to examine the possibility of generation of resistance against the novel compounds under test. The latter can be done by investigating the efficacy of the antileishmanial agent against resistant strains. Moreover VL is a disease related to the severe suppression of immune system of the host. Thus it is indispensable that the leishmanicidal agent should exhibit good immunostimulatory properties.

In this context the present study deals with the investigation of antileishmanial agent which could overcome the shortcomings encountered during treatment of VL especially immunosuppression, toxic effects and drug-resistance. The present study examined the immunomodulatory and antileishmanial efficacy of plant extract of Rhodiola imbricata and its two bioactive compounds namely salidroside and rutin. Two distinct strains of L. donovani presenting different sensitivity towards SSG have been used in the studies to confirm the broad range efficacy of the plant extract and its active compounds. The testing of plant extract and the compounds was initiated with the in vitro studies to check their antileishmanial activity by trypan blue dye exclusion test and cell cycle analysis through PI staining. Further the in vivo potential of plant extract and the compounds was checked in the inbred BALB/c mice in terms of LDU and real-time PCR. Moreover the immune status of the host was analyzed after treatment of the infected animals with HERERI, SAL or RTN through DTH reaction in response to leishmanin antigen, levels of different Th1/Th2 cytokines, population of CD3+, CD4+, cytotoxic CD8+ T cells and CD 19 B cells. In addition to this the effect of active bio-compounds at the transcription level was checked by quantifying
the levels of NF-κB and iNOS genes. The ability of active compounds to generate leishmanicidal molecules, NO and ROS was also tested. Further the safety of these compounds was also checked in the BALB/c mice by biochemical and histological methods.

*Rhodiola imbricata* Edgew. is a high altitude plant which has wide medicinal properties. The hydro-ethanolic extract of *R. imbricata* revealed the presence of many phytochemicals including phenols, flavonoids, alkaloids, flavonol glycosides, diterpenes and triterpenes and it was found to be a rich source of flavonoids, terpenoids and phenols. Two pharmacologically important bioactive components i.e. salidroside (SAL) and rutin (RTN) were detected in the plant extract. SAL is a phenylpropanoid glycoside while RTN is a flavonoid endowed with great curative and therapeutic activities.

HERERI, SAL and RTN depicted effectual *in vitro* activity against the promastigotes of *L. donovani* coupled with lack of any cytotoxicity effects on THP-1 cells. Out of the three tested agents, RTN exhibited promising inhibitory activity against not only the sensitive but resistant strain of *Leishmania donovani*. The resistant strain possesses higher power of reproducibility and hence fitness than the sensitive strain. The R-strain parasites are well adapted to survive even in the presence of lethal dose of drug. Inspite of this resistant behavior of parasite RTN inhibited the growth of R-strain promastigotes. This suggested the competent antileishmanial activity of RTN. The findings were further confirmed by exploring the effect of these agents on the cell cycle of parasite. As expected HERERI, SAL as well as RTN arrested the promastigotes at sub G0/G1 phase. However the number of resistant parasites in the sub G0/G1 phases was found to be maximum in case of RTN treated promastigotes. It was concluded that RTN exhibited inhibitory effects against sensitive as well as resistant parasites due to its apoptotic potential.

After the encouraging results in the *in vitro* system the study was further advanced and antileishmanial efficacy was monitored in inbred BALB/c mice. HERERI, SAL and RTN decreased the parasite load in the infected animals. In contrast to HERERI, the active compounds i.e. SAL and RTN showed more reduction in the parasite load in animals infected with either S- or R-strain. The resistant
parasites show more tolerance towards drugs via number of means including changes in the membrane drug transporters. However approximately similar level of decrease in the parasite load was obtained in animals infected with either S- or R-strain by SAL and RTN. This indicated that SAL and RTN can account for diverse parasite strains in terms of drug sensitivity as these active compounds overcame the problem of reduced uptake of drug in case of resistant parasites too.

A marked increase in the DTH response was attained after HERERI, SAL and RTN treatment. However SAL and RTN exhibited heightened DTH response which was more than HERERI not only against S- but also against R-strain parasites. Although the resistant parasites handicap the host in terms of eliciting the cell mediated mechanisms yet SAL and RTN successfully generated DTH response in animal model where infection was induced by resistant parasites. This proved the immunomodulatory potential of SAL and RTN in the host irrespective of type of strain involved.

The effectiveness of chemotherapy relies on the restoration of defective immune response. HERERI, SAL and RTN skewed the imbalanced Th1/Th2 response in the infected mice. The levels of IFN-γ, TNF-α and IL-12 were found to be enhanced after treatment with plant extract and its active compounds proposing their immunostimulatory property. The generation of Th1 cytokines was higher in SAL and RTN treated infected groups than the HERERI treated animals. Moreover SAL and RTN were found to be equally effective in generating Th1 cytokines in murine model irrespective of infecting strain involved. The resistant parasites are responsible for more grievous outcome of infection by severely impairing the T-cell response specific to *Leishmania* than the sensitive strain. However SAL and RTN counter the resistant environment adequately by modulating the immune system to produce higher amount of key cytokines of Th1 type.

The animals showed pronounced Th2 tilted immune status after infection as evidenced by higher levels of IL-10 and IL-4 cytokines. After HERERI, SAL or RTN therapy lower secretion of Th2 cytokines was experienced in the infected animals. However in comparison to HERERI, the SAL and RTN treatment resulted in minimal production of IL-10 and IL-4 in the animals infected with either strain. The higher
Th2 and lower Th1 response confers resistance to the parasite from the drug pressure and thus the treatment failure. SSG is well known example for this kind of resistance. SAL and RTN potentially controlled the Th2 dominant environment of immune system even in the R-strain infected animals. This suggested that these compounds are responsive even towards the resistant phenotype.

During infection of *L. donovani*, suppressed expansion of protective CD3$^+$, CD4$^+$ and CD8$^+$ T cells is commonly recorded. However HERERI, SAL and RTN inherently augmented the proportion of these splenic T-cell subsets. This may be attributed to the immunopotentiating effects of the plant extract and the natural bioactive compounds. While HERERI upregulated the number of CD3$^+$, CD4$^+$ and CD8$^+$ T lymphocytes in S-strain infected group, these changes were less evident in R-strain infected animals. However SAL and RTN increased the IFN-γ producing CD4$^+$ as well CD8$^+$ T lymphocytes in S- as well as R-strain infected animals. This reflected that the bioactive compounds steered the therapeutic cure by increasing the population of T lymphocytes. Moreover the efficacy of SAL and RTN in R-strain infected animals in generating T-cells highlighted their strong ability to revert the resistance.

CD19 B cells contribute in the disease pathogenesis during *L. donovani* infection. There is direct relation between the expansion of B cells and disease progression. The plant extract and its bioactive components decreased the augmented number of these cells. HERERI, SAL and RTN controlled the VL infection by reducing the number of CD19 B cells. This suggested that modulation of the activation and population of CD19 B cells during infection may serve as efficacious treatment strategy.

When the levels of expression of genes viz. iNOS and NF-κB were determined in animals infected with either strain, it was found to be down-regulated. However SAL and RTN increased the expression of these genes in spleen of animals infected with either strain as compared to the untreated infected animals. The R-strain parasite expresses its resistant behavior by shunting the expression of many genes including iNOS and NF-κB. The latter genes are crucial for generation of NO, ROS and Th1 immune response in the host. The reduction in parasite load
following treatment with bioactive compounds was accompanied by increase in the expression of these important genes, iNOS and NF-κB not only in S- but also in R-strain infected animals. This indicated that the exposition of the resistant parasites to the active compounds resulted in reversal of resistance and these compounds can be exploited further in the high-throughput drug screening tests.

The therapeutic implication of SAL and RTN in VL infection was further checked by their ability to induce the generation of microbicidal agents like NO and ROS. The treatment of SAL and RTN resulted in potent induction of toxic leishmanicidal intermediates of oxygen in both SSG susceptible and –resistant strains of *L. donovani* infected BALB/c mice. The findings suggested that these active compounds could control and diminish the parasite burden by generating the oxidative stress disadvantageous for the parasite. Moreover the R-strain parasites could survive better even in NO and ROS rich environment. In addition resistant parasites prevent the production of these molecules in host through number of mechanisms. However the increased levels of NO and ROS observed even in the R-strain infected animals after SAL or RTN treatment advocated the ability of these active compounds in reversing the drug resistance. From the findings it can be surmised that the immunological pathway followed by SAL and RTN that controlled the VL infection involves the production of NO via activation of iNOS gene and other toxic reactive oxygen species.

Hepatomegaly and renal dysfunction are one of the characteristic symptoms during VL infection. The impaired functions of liver and kidney were observed in the infected animals of either strain as the levels of biomarkers of both these organs were noticed to be in the abnormal range. Similar outcome was observed in the infected animals of either strain after SSG and AmB treatment. The SSG treated animals’ depicted hepatic disorders while AmB treated animals exhibited renal impairment. However HERERI, SAL or RTN treatment restored the increased abnormal levels of liver as well as kidney function biomarkers in the infected animals of either strain. The findings revealed the inert nature of plant extract, SAL and RTN against toxicity of either liver or kidney. In lethal dose studies also, HERERI did not compromise the viability of animals even at the dose of 5 g/kg b.wt.
Further the histological studies of liver, kidney and spleen in the infected animals of either strain revealed the destructive influence of *L. donovani* infection on these organs. The hepatotoxicity observed in terms of abnormal liver enzymes was also observed at the histological studies after the SSG treatment. Likewise although no effect of AmB was observed on the liver but kidney histology was shown to be damaged. This further supported the hepato- and nephro-toxicity of SSG and AmB respectively. The HERERI, SAL or RTN treatment attenuated the histopathological outcome characterized by normal sections of liver, kidney and spleen. This further demonstrated the safety of the plant extract and its bioactive components. Presently when conventional chemotherapeutic options are facing the threat of resistance and escalate severe side-effects in the host, the discovery of safe antileishmanial agent which exhibits less or no possibility of evolution of resistance is highly recommended. The switching of chemical based drugs to the compounds obtained from plants seems to be an impressive approach as they have minimal side-effects. Moreover large body of literature supports the efficacy of plants in the field of antileishmanials.

The present study clearly demonstrated the therapeutic properties of HERERI, SAL and RTN in terms of immunostimulation and direct leishmanicidal effects. The mechanism of action of tested agents was unraveled from the findings of the present study. SAL and RTN illustrated encouraging antileishmanial potential substantiated by its Th1 stimulating and Th2 suppressing efficacy. SAL and RTN increased the expression of iNOS and NF-κB genes which are directly involved in the generation of NO, ROS and Th1 immune responses in the host. Moreover the tested agents controlled the growth of parasite without adversely affecting the host.

Furthermore very importantly the study also highlighted the importance of SAL and RTN in drug discovery pipeline in terms of evolution of resistance. The study revealed that the compounds of HERERI are active against both SSG sensitive as well as resistant *Leishmania donovani* strains. It depicted the broad range efficacy of these compounds and warrants their ability to enter in the armamentarium of the available antileishmanials.
The following conclusions can be drawn from the observations made in the study:

1. HERERI was found to be a great source of different phytochemicals like phenols, flavonoids, alkaloids, flavonol glycosides, diterpenes and triterpenes.

2. SAL and RTN were identified by LC-MS and HPLC respectively.

3. HERERI, SAL and RTN depicted good inhibitory potential against SSG-resistant and sensitive strain of *L. donovani*. Among all tested agents RTN exhibited lowest and approximately equivalent IC$_{50}$ against sensitive as well as resistant strain.

4. No cell cytotoxicity of HERERI, SAL and RTN was documented and the tested agents were found to be more selective against parasites than the human cells.

5. The administration of HERERI (LD$_{50}$>5g/kg) induced no mortality or behavioral changes in mice.

6. HERERI, SAL and RTN arrested the promastigotes at the sub G$_0$/G$_1$ phase. RTN was most effective in causing cell cycle arrest in both sensitive and resistant promastigotes.

7. HERERI, SAL and RTN decreased the parasite burden in liver and spleen of inbred BALB/c mice.

8. HERERI, SAL and RTN heightened the DTH response in infected animals. Enhanced cell-mediated immunity was generated after SAL or RTN treatment in sensitive as well as resistant strain infected animals.

9. The immune status was tilted more towards the Th1 type after HERERI, SAL and RTN treatment. SAL and RTN were more efficient in increasing the titres of Th1 cytokines in mice infected with either sensitive or resistant strain.

10. The population of different sub sets of T cells viz. CD3$^+$, CD4$^+$ and CD8$^+$ T lymphocytes was found to be expanded after HERERI, SAL or RTN treatment. SAL and RTN were most functional in generating these T cells in animals infected with either strain. Additionally, the plant extract and the active compounds effectually decreased the number of CD19 B cells.
11. SAL and RTN upregulated the expression of iNOS and NF-κB genes and also increased the reactive oxidative leishmanicidal molecules viz. NO and ROS in animals infected with the either strain.

12. No treatment related side-effects were diagnosed in HERERI, SAL or RTN treated infected animals as normal levels of biomarkers of kidney and liver functions were assessed along with the normal histology of kidney, liver and spleen (Fig. 6.1).

**Future perspectives**

In future the studies should be carried out on the higher animal models. Additionally more studies are required to be done in other species and strains of *Leishmania*. 
FIG. 6.1: SUMMARY

Leishmania donovani

Cell cycle analysis

Arrest at Sub G₀/G₁ phase

Leishmania donovani

Salidroside/ Rutin

Increased DTH response

Reduction in the parasite load

Parasite killing

NO, ROS

IFN-γ, TNF-α, IL-12

iNOS GENE

NF-κB GENE

CD4

CD8