DISCUSSION

Visceral leishmaniasis falls in the neglected diseases as it is the problem mainly of the poor people in developing countries. The pharmaceutical companies scarcely pay heed to the development of drugs for the treatment of this parasitic disease as they find no monetary profits from it (Varela-M et al., 2012; Bi et al., 2018). Many researches are actively focused on finding an alternative therapy from the medicinal plants. Infact, approximately about 25% of the drugs available in the market have been obtained directly or indirectly from the plants (Vila-Nova et al., 2013). The secondary metabolites which play an important role in the plants serve as a source of active molecules for the preparation of novel antileishmanial agent (Oryan, 2015). Further the fight against VL can only be triumphed by the discovery of drug which possesses leishmanicidal activity along with great immunomodulatory potential. In addition a safe and an effective drug especially against the drug-resistant strains is very essential because growing drug resistance is the major risk factor in drug development. So the efficiency of antileishmanial agents should be confirmed on the grounds of danger of emergence of resistance beforehand (Hefnawy et al., 2017). Moreover the practice of therapeutics to treat leishmaniasis can make success only in the presence of active immune system. Thus one of the approaches in the drug discovery pipeline to treat leishmaniasis depends upon the therapy which upregulates the immune status and is deprived of any side-effects in the host (Chouhan et al., 2015). The immunomodulators procured from plants are affordable, safe and have the efficacy to maintain homeostasis of the immune system throughout the course of prolonged treatment regimen (Gultierrez-Rebolledo et al., 2017).

Plants of *Rhodiola* genus are predominantly exploited for their invaluable medicinal properties. The present study interrogated the antileishmanial activity of hydroethanolic root extract of *R. imbricata* Edgew. (HERERI) and its bioactive components, Salidroside (SAL) and Rutin (RTN) for the first time in comparison to two reference drugs, AmB and SSG. The efficacy of HERERI, SAL and RTN was tested against SSG-sensitive and resistant strains of *L. donovani* infected inbred BALB/c mice.

In phytochemical tests, HERERI showed positive results to phenolic compounds, flavonoids, alkaloids, flavonol glycosides, diterpenes and triterpenes.
Further SAL, a phenylpropanoid glycoside was analysed by LC-MS and compound, RTN which is a flavonoid was uncovered by HPLC method. Our data matched with the previous analysis in which SAL and RTN were confirmed in *R. imbricata* by LC/MS and HPLC respectively. Likewise GC/MS assay reported the presence of many secondary compounds like terpenes, flavonols, alkaloids, phenols in the plant which renders multifarious pharmacological properties to this plant (Yu et al., 2008; Tayade et al., 2013; Senthilkumar et al., 2014). After achieving convincing qualitative phytochemical screening results, the quantitative analysis of flavonoids, terpenoids and phenols was performed. HERERI was found to be a rich source of these chemical compounds. There are many reports describing the powerful antileishmanial properties of these flavonoids (Ogeto et al., 2013), terpenoids (Sousa et al., 2014) and phenols (Araujo et al., 2018).

In the present study the growth inhibitory action of HERERI, SAL and RTN was examined against the promastigotes of SSG responsive as well as SSG unresponsive strain of *L. donovani* in terms of IC$_{50}$. The effect of HERERI, SAL and RTN on the parasite was determined by trypan blue dye exclusion test. The plant extract as well as the active compounds exhibited toxicity against *L. donovani*. The treatment of either strain of *L. donovani* with HERERI or its active compounds demonstrated suppression of parasite growth in a dose dependent manner. The anti-promastigote activity of HERERI and SAL was more against the sensitive strain than against the resistant strain. However RTN exhibited similar effect against sensitive (IC$_{50}$=12.64±0.86 µg/mL) as well as resistant (IC$_{50}$=13.07±1.42 µg/mL) strain. Similar study conducted by Guimarães et al., 2013 showed that naphthoquinoidal compounds have potent antileishmanial activity against both antimony-sensitive and resistant promastigotes of *L. infantum* and *L. amazonensis* with low IC$_{50}$ and high selectivity index. The study of Kaur et al., 2010 found that hexane, ethyl acetate and chloroform extract of rhizomes of *Alpinia galanga*, displayed anti-promastigote activity *in vitro*. Similarly Di Giorgio et al., 2004 showed that harmane, an alkaloid exerted strong antileishmanial activity. It arrested the promastigotes at the S-G$_2$M phase and on the other hand prevented the amastigotes to enter the macrophages.

The determination of cytotoxicity of the plant extract, SAL and RTN against human cells is very crucial to obtain the selectivity profile against *L. donovani*. To test
the cytotoxicity of HERERI, SAL and RTN, cells were incubated with different concentrations of extract and the active components. The viability of these cells was determined by MTT reduction assay after 48 hrs. The 50% cyto-toxicological effect (CC$_{50}$) of tested extract and compounds was scrutinized against human monocytic leukemia cell line (THP-1). The CC$_{50}$ was found to be within the acceptable toxicity limit accompanied by a high selectivity index (more than 10) in all tested agents. The findings indicate that HERERI, SAL and RTN are more selective in killing the promastigotes than the human cells. Previous studies have also found comparable cytotoxic status (CC$_{50}$>1000 µg/mL) of RTN when tested on the Vero cells (Zandi et al., 2011). In a study by Tavares et al., 2018 quinoline derivative showed inhibitory action against the amastigotes as well as promastigotes of *Leishmania*. The derivative was found to have no toxic effect on the macrophages of mice and RBCs of human with SI greater than 10.

Further it was very crucial to test the side-effects of HERERI in the *in vivo* model too. The lethal dose of HERERI was estimated according to the method of Lorke. No signs of toxicity were observed after oral administration of HERERI at the highest dose of 5 g/kg b.wt. The presence of normal liver and kidney histology and absence of any death among the HERERI treated animals suggested the wide safety index of the plant extract. This observation is in harmony with the study of Gupta et al., 2008 who proposed that the aqueous extract of roots of *R. imbricata* is safe for consumption at the dose >10 g/kg/b.wt.

Further the effect of plant extract and its bioactive components on the cell cycle of *Leishmania* parasite was tested. Flow cytometric analysis was performed to enumerate the percentage of cells in different stages of cell cycle. The parasite cells were labeled with PI after fixation and permeabilization with ethanol. The amount of bound PI dye was elucidated by fluorescence which corresponded to the content of DNA. The intensity of fluorescence lesser than that of cells present in G$_{0}$/G$_{1}$ cells expressed the cells in the sub-G$_{0}$/G$_{1}$ phase i.e. in the apoptotic condition with fragmented DNA (Sen *et al.*, 2007; Ahuja *et al.*, 2018). In the current study the promastigotes of the SSG sensitive and resistant strain were treated with the IC$_{50}$ dose of HERERI, SAL and RTN for 72 hrs. HERERI and SAL were more effective against
the promastigotes of S-strain as compared to the R-strain. However after incubation with RTN almost equal proportion of SSG responsive as well as SSG-unresponsive parasites were noticed in the sub-G_0/G_1 phase. The ability of the plant extract and its active compounds to check the cell cycle advocated their capacity to attenuate the parasite growth by altering the normal cell cycle. In addition the capacity of SSG to halt the R-strain parasites at sub-G_0/G_1 phase was obsolete as compared to RTN. The potential of RTN to even arrest the R-strain promastigotes presented its utility in resistant cases also. Similar assay was used in the work of Sen et al., (2007) in which the effect of antileishmanial agent, artemisinin (IC_{50}=160 mM) was checked on the cell cycle of promastigotes. Artemisinin was found to increase the apoptosis of cells after 48 hrs of incubation of parasite resulting in 33.11% of cells in the sub- G_0/G_1 phase as compared to only 3.03% in control cells in the same phase (Sen et al., 2007). The biofractions of Azadirachta indica reduced the multiplication of the parasite by triggering apoptosis characterized by appearance of the cells in the sub-G_0/G_1 phase. The phenolic component of turmeric, curcumin arrested Leishmania cells at the sub-G_0/G_1 phase with increase in the concentration of levels of calcium in cytoplasm and dissipation of membrane of mitochondria (Chauhan et al., 2018). Similarly racemoside A, a steroidal saponin was found to exert its antileishmanial potential by arresting the promastigotes of L. donovani at the sub- G_0/G_1 phase (Dutta et al., 2007). The results obtained from the in vitro system depicted the growth inhibiting ability of HERERI, SAL and RTN against the promastigotes of L. donovani. This further prompted us to investigate them in the in vivo system to delineate their effect in the biological system.

Inbred BALB/c mice were chosen to examine the antileishmanial potential of plant extract and its bioactive compounds. The mice were infected intravenously with the promastigotes of L. donovani. After 30 days of infection the mice were treated with HERERI (500 and 1000 mg/kg b.wt.), SAL (25 mg/kg) and RTN (25 mg/kg) for 14 days. Further the parasite load was calculated by giemsa stained impression smears and by SYBR green based real time PCR on 7 and 14 p.t.d. HERERI was found to be more effective at the higher dose against SSG sensitive as compared to the SSG resistant strain of L. donovani. However it was notable that SAL and RTN decreased
the parasite burden in the S- as well as R-strain infected animals. SAL and RTN were found to be approximately equally effective in resolving VL infection caused by either sensitive or resistant parasites on 7 as well as 14 p.t.d. On the other hand, SSG treatment at the dose of 40 mg/kg showed significant clearance of sensitive but not resistant parasites. The failure of SSG to clear the resistant parasites is not surprising as resistant parasites upregulate ABC transporters in the membrane of host cells which cause the efflux of drug, SSG. The resistant parasites even exhibit defective uptake of the drug (Shaked-Mishan et al., 2001; Basu et al., 2008). The efficiency of SAL and RTN to treat the infected animals infected with even resistant parasites confirmed their desirable internalization and their satisfactory retention in infected cells. It also illustrated the capacity of the active components to endure and reverse the drug resistance mechanisms. In a similar study it was found that the administration of crude extract of *Momordica charantia* (300 mg/kg b.wt.) and its active purified component, Momordicatin (10 mg/kg b.wt.) in hamster with VL infection resulted in 100% clearance of the parasite (Gupta et al., 2010). The efficacy of a synthetic compound namely, pentalinonsterol to treat VL in BALB/c mice was assayed. At the dose of 2.5 mg/kg b.wt. it reduced the parasite burden in the liver and spleen (Gupta et al., 2015). Similarly compounds like pleiocarpin, luteolin, buchtienin and quercitin have been found to be active against *L. donovani* (Gultierrez-Rebolledo et al., 2017).

The immunological outcome after the administration of HERERI and its active components was tested by estimating the DTH response, proportion of different T cells, Th1/Th2 cytokines and expression of inducible NOS and NF-κB transcription factor genes.

Delayed-type of hypersensitivity reaction (DTH) is interposed by the Th1 fraction of CD4+ T cells with the dissipation of the same during the serious episode of VL (Singh et al., 2012; Stober et al., 2012). Many studies reinforce the view that the generation of DTH is a supreme marker for the development of resistance against infection (Jeronimo et al., 2004). The current study monitored the magnitude of foot swelling after 48 hrs of leishmanin injection and revealed the generation of pronounced DTH reaction post treatment in all groups of animals as compared to the infected controls. HERERI treatment showed increased DTH reaction in the infected
animals as compared to the infected controls on both p.t.d. In the prior studies too *R. imbricata* has been evaluated for its ability to evoke DTH response in rats. *Rhodiola* enhanced the antibodies specific to the antigens like tetanus toxoid and ovalbumin and thus aroused heightened DTH reaction (Mishra *et al*., 2010). In the present study it was noted that HERERI at the higher dose mounted an increased delayed hypersensitivity response in S-strain infected animal as compared to the R-strain infected mice. However SAL and RTN treatment in S- as well as R-strain infected animals substantially evoked strong DTH reactivity on both p.t.d. The increase in the right footpad thickness as compared to the left footpad of infected animals after administration of SAL and RTN exhibited the ability of these bioactive compounds to enhance the population of infiltrating macrophages at the site of inflammation. The findings of the present study elucidated the capacity of active components to control VL infection by induction of specific cellular immunity irrespective of the strain involved in infection. The previous study of Ganeshpurkar and Saluja, 2017a proved the immunomodulatory potential of RTN in a rat model. The administration of RTN increased cell mediated immune mechanisms as depicted by enhanced DTH reaction induced by sheep RBCs. RTN was also found to restore the efficacy of leucocytes in rats treated with cyclophosphamide. In the study of Chouhan *et al*., infected BALB/c mice after treatment with the ethanolic fractions of leaves and seeds (200 mg/kg b.wt.) of *A. indica* exhibited strong DTH response (Chouhan *et al*., 2015). In another study therapeutic effect of oral administration of n-hexane fractions of leaves and seeds (200 mg/kg b.wt.) of *Artemisia annua* against murine VL was accompanied by heightened DTH response (Islamuddin *et al*., 2015). The study of Abid *et al*., 2012 registered that ethyl acetate fraction of fruits of *Prunus cerasus* (50 mg/kg) decreased the parasite load and increased DTH reaction in infected BALB/c mice. In another study *Bergenia ligulata* was observed to provide long lasting immunogenicity in *L. donovani* infected mice after treatment with 500 and 1000 mg/kg b.wt. of the plant extract. The plant extract was found to establish strong T-cell response as depicted by enhanced DTH reaction (Kaur and Kaur, 2018).

In the current study analysis of different subsets of lymphocytes was performed using flow cytometry. Total percentage count of CD3+, CD4+, cytotoxic
CD8$^+$ T cells and CD19 B plasma lymphocytes was quantified on the 14 post treatment day in different groups of animals. The polarization of the cell profile during VL is mediated via cytokines like IL-12 and IL-4. IL-12 modulates the differentiation of CD4$^+$ T lymphocytes towards Th1 immune profile. However IL-4 differentiates the CD4$^+$ T cells towards the Th2 type. The latter further suppresses the NO secretion and favors the survival of parasite (Rodriguez and Wilson, 2014).

CD4$^+$ T cells exhibit an important function in constructing dominant anti-parasitic immunity by directing the immune surveillance towards restricting the growth of parasite. These cells are the main producer of IFN-γ which plays important role in controlling the infection (Kumar et al., 2014).

The individuals resistant to VL express high levels of CD8$^+$ T cells. These cells heal VL even in the individuals with prolonged record of disease. The CD8$^+$ T cells contribute to the protection via their cytotoxic activity which is stimulated through the pathway of perforin-granzyme B protein (Kaushal et al., 2014). These cells also lead to the recruitment of inflammatory cells and also play important function in sustaining the granuloma formation during VL (Belkaid et al., 2002). It plays protective role in murine as well as human VL caused by L. donovani or L. infantum. These cells are found to bestow resistance to the re-infection and thus imply its possible use in the development of vaccine (Rossi and Fasel, 2018).

In the present study it was observed that after infection with L. donovani of either strain the percentage proportion of CD3$^+$, CD4$^+$ and cytotoxic CD8$^+$ T cells is decreased. This is in accordance with the previous study of Roy et al., which described that parasite evades the immune system by modulating the expression of inhibitory receptor, programmed death (PD) 1 on the CD4$^+$ T cells and thus results in the exhaustion of T cell population during VL (Roy et al., 2017b). Moreover infection of L. donovani decreases the proliferative potential of CD4$^+$ and CD8$^+$ T cells and also conditions these cells to switch to the Th2 immune phenotype (Nylen and Gautam, 2010).

Therefore the success of antileishmanial therapy depends on the elicitation of effective repertoire of T cells in the spleen. In the current study it was observed that the concentration of these cells was much lower in the animals after infection with R-
strain than in the animals infected with S-strain. This was in agreement with the previous study of Mukherjee et al., 2012 which stated that the resistant parasites survive better in the host as compared to the sensitive one by hampering the activation of parasite specific T cells. In the present study HERERI, SAL and RTN elicited marked production of CD3\(^+\), CD4\(^+\) and cytotoxic CD8\(^+\) T cells in the infected animals. The study revealed that HERERI at a higher dose is more effective in increasing the number of these cells in the S-strain infected animals as compared to the R-strain infected animals. However SAL and RTN treatment significantly increased the population of CD3\(^+\), CD4\(^+\) and CD8\(^+\) T cells in the spleen of animals infected with either sensitive or resistant strain. As expected the treatment of SSG could not increase the number of these cells in animals infected with resistant strain. This observation is relevant in terms that SAL and RTN are capable of reversing the resistant phenotype by augmenting the percentage of different T cells in the spleen of animals infected with resistant parasites. Previous studies supported influential property of SAL on the population of different subsets of T cells. The supplementation of SAL (24 mg/kg of b.wt.) in the diet of aged mice (21 months) increased the total T cells (CD3\(^+\)) and T helper cells (CD4\(^+\)) in the spleen (Lu et al., 2013). Moreover it also augmented the percentage of CD4\(^+\) and CD8\(^+\) lymphocytic population in the splenocytes of mice when administered along with OVA governing its competent adjuvant activity (Guan et al., 2011).

According to previous studies T cell-mediated immune response portrays crucial role in controlling VL. Various other studies prove the function of CD3\(^+\), CD4\(^+\) and CD8\(^+\) T cells in restricting the growth of L. donovani in animal model. For instance the therapeutic efficacy of triterpene, astrakurkurone (Mallick et al., 2016) has been found to be induced by the multiplication of CD4\(^+\) T cells in the spleen. Similarly the phenotypic analysis revealed that bioactive fractions of Piper nigrum (200 mg/kg) elevated the CD4\(^+\) and cytotoxic CD8\(^+\) T cell percentage population in the infected mice (Chouhan et al., 2015). The study of Islamuddin et al., 2015 evaluated the antileishmanial activity of extract of Artemisia annua in L. donovani infected BALB/c mice. It was found that the plant extract controlled the VL infection by increasing the proportion of CD4\(^+\) and cytotoxic CD8\(^+\) T cells in the spleen. The
extract was found to enhance the proliferation of lymphocytes and up-regulation of molecules like CD80 and CD86.

Further the present study quantified the percentage of CD19 B plasma cells in the spleen of different animals. The mature B cells in mouse exhibit CD19 surface markers. In the peripheral lymphoid organs the mature B cells play role in producing immunoglobulins mainly IgM upon interaction with antigen (Lund and Randall, 2010). In addition according to previous studies B cells have been reported to produce a potent inhibitor of macrophage, i.e. IL-10 (Fillatreau et al., 2002) and TGF-β (Parekh et al., 2003). B cell-derived IgM and IL-10 exhibit detrimental role as it results in the VL susceptibility (Deak et al., 2010; Bankoti et al., 2012). The amastigotes of *L. donovani* activate B cells by inducing endosomal toll like receptors and exacerbate the disease by promoting hypergammaglobulinemia and anti-inflammatory cytokines (Silva-Barrios et al., 2016). The prior studies advocate no role of antileishmanial specific antibodies in the amelioration of disease; instead on the other hand these antibodies favor the establishment of infection. Mutant mice with deficient mature B cells displayed better defiance against *L. donovani* (Smelt et al., 2000). The individuals of VL endemic regions showed negative relationship between the levels of antibody and DTH response. However, a very high affirmative correlation was encountered between high levels of anti-leishmanial antibodies and embellished disease (Miles et al., 2005). In the present study the infected animals depicted the expansion of CD19 B cells. Similarly Deak et al., 2010 observed population of B cells to be increased by 14-folds after 1 month of infection. In the current study the population of B cells decreased after treatment of infected mice with HERERI, SAL or RTN in S- as well R-strain infected animals. This proved the contribution of plant extract and its bioactive components in decreasing the disease pathology via declining the B cell population and thus deactivating the induction of polyclonal B-cell response.

During VL cytokines like IL-12, 4, 10, IFN-γ and TNF-α exhibit pivotal contribution in the suppression or growth of the disease. Thus in the current study the levels of different lymphokines associated with two poles of helper T cells designated as T helper type 1 (Th1) and Th2 were estimated after 7 and 14 p.t.d. in the sera
samples of different groups of animals. Th1 type is involved in the production of IL-12, TNF-α and IFN-γ while Th2 cells produce IL-4 and IL-10. Thus quantification of these cytokines marks the status of the respective subset of helper T cells (Gurung and Poudel, 2018). The cytokines, IFN-γ and TNF-α triggers the microbicidal activity of the macrophages, while conversely IL-10 and IL-4 result in deactivation of the activity of macrophages (Goto and Prianti, 2009). There is an impressive range of evidences supporting that induction of Th1 along with the impediment of Th2 phenotype results in the abolishment of the VL and thus signifying the outcome of treatment (Oliveira et al., 2014). In the present study experimental VL model exhibited marked distinct profile of cytokines in the sera as compared to treated mice. In the infected animals *Leishmania* parasite modulated the immune response in its favor as revealed by increased Th2 immune response. Similar outcome of the infection was found in the study of Suman et al., 2018 where the protozoan, *Leishmania* was found to thwart the immune defenses of the host by eliminating protective Th1 immune response. The study revealed that tryparedoxin protein progresses the VL infection by downregulating the host Th1 response and favoring IL-4 and IL-10 producing Th2 cells. In this respect in the present study also the titres of Th2 cytokines were found expanded in the serum of only infected animals with diminished levels of Th1 related cytokines. However substantial immunity was encountered upon treatment of all groups of animals after 7 as well 14 p.t.d.

Previous studies correlate the elevated levels of IFN-γ with the control of parasite and absolution from the disease while the neutralization of IFN-γ impairs the host’s ability to limit the infection (Singh and Sundar, 2018). Similarly the therapeutic outcome of HERERI and the active components was found to be correlated to the development of IFN-γ dominant immune status in the sera of S-strain as well as R-strain infected animals. IFN-γ therapy holds a key therapeutic role in terms of the management of VL in the individuals suffering from idiopathic CD4⁺ T cell lymphocytopenia (Sternfeld et al., 2010). The previous study involving the treatment of *Allium sativum* during the experimental leishmaniasis revealed the similar findings. *A. sativum* controlled the infection by increasing the secretion of IFN-γ and NO in the infected animals (Gamboa-Leon et al., 2007). Likewise the study of Mallick et al., 2016 showed that triterpene, astrakurkuron of *Astraeus hygrometricus* controlled the
VL infection in mice by increasing the levels of IFN-γ in the serum. Another plant, *Kalanchoe pinnata* at the dose of 400 mg/kg for 30 days reduced the parasite burden in infected BALB/c mice. The protection was imparted by increasing the levels of IFN-γ and NO (Gomes et al., 2010).

IL-12 is known to induce IFN-γ via interaction with T cells and NK cells. It thus plays important role in subsequent development of Th1 immune responses (Messlinger et al., 2018). It is also a key player in differentiating the naive Th cells into Th1 cells (Athie-Morales et al., 2004). The study of Bacellar et al., 2000 found that the neutralization of IL-12 inhibits lymphoproliferation. The study also depicted that IL-12 plays significant role in maintaining the capacity of VL patients after treatment in producing IFN-γ. Further the study of Novais et al., 2018 showed that even the CD8⁺ T cells fail to secrete IFN-γ in the deficit of IL-12. In the present study HERERI, SAL and RTN was found to possess Th1 driving activity as their treatment increased the levels of IL-12 in the sera of infected animals on 7 as well as 14 p.t.d.

In the present study another Th1 cytokine, TNF-α which is a protein produced largely by macrophages was also estimated in the sera of different animals. It possesses extensive biological activities including inflammatory processes. TNF-α has been tethered to the reign over VL infection as it activates macrophages and granuloma development (Engwerda et al., 2004). This cytokine acts alongwith IFN-γ and kills the intracellular parasite by induction of NO (Nashleanas and Scott, 2000). The blockade of TNF-α interferes with the secretion of IFN-γ and lead to the disease severity (Singh et al., 2016). In the present study HERERI, SAL and RTN was found to enhance the secretion of TNF-α in the sera of infected animals on both p.t.d. The findings of the previous studies support the immunomodulatory property of *R. imbricata*. The study of Mishra et al., concluded that *R. imbricata* resulted in the enhancement of immune response by the activation of intermediary proinflammatory molecules via phosphorylation of transcription factor NF-kappa B and inhibitory-kappa B. Following this mechanism of action it resulted in production of TNF-α in human PBMCs and mice RAW 264.7 cell line (Mishra et al., 2006). Similarly there are many previous studies which also reinforce the immunomodulatory efficacy of SAL. It showed considerable humoral and cellular immunological responses in mice.
when co-administered along with ovalbumin (OVA). Co-administration of SAL with OVA enhanced the proliferation of splenocytes and promoted the secretion of cytokines like IL-2 and IFN-γ to much higher titers than in only OVA treated animals (Guan et al., 2011).

The animals infected with the R-strain are more difficult to treat as the R-strain parasites are known to hamper the immune system more severely than the S-strain parasites. The resistant parasites critically suppress the Th1 immune status by inducing low expression of TNF-α, IFN-γ and IL-12 (Mukherjee et al., 2013). In the present study a strong IL-12 environment driven by IFN-γ and TNF-α was triggered by SAL and RTN in all treatment groups irrespective of the strain of *L. donovani* involved. This finding suggests that the therapeutic role of SAL and RTN in murine VL infection is attributed to their ability to attune the immune status in the benefit of host even after infection with resistant parasites.

Various other studies support that stimulation of Th1 response could control the VL infection. For instance the study of Dey et al., 2015 demonstrated that the hexane extract of *Croton caudatus* decreased the parasite burden in dose-dependent manner. The oral administration in the infected mice for 5 days resulted in immunostimulation by induction of Th1 over the Th2 immune response. Similarly the extracts of various other plants like *Xylopia discreta* (Lopez et al., 2009), *A. indica* (Dayakar et al., 2015), *Solanum* spp. (Cos et al., 2018), *Desmodium gangeticum* (Mishra et al., 2005) have been found to control the infection of *L. donovani* by immunomodulation in terms of increment in the Th1 immune response.

The severity of disease is associated with the hampered activation of T-cells and high expression of suppressive IL-10 and IL-4 cytokines (Stager et al., 2003; Mesquita et al., 2018). Thus in the current study the levels of these important cytokines were tested in the sera of different animals.

IL-10 has been identified as an important cytokine not only for the development of disease, but also for the successful persistence of parasite in macrophages (Mukherjee et al., 2012). IL-10 cripples the immune status in broad ways and thus disables it to control the infection. It encourages the parasite survival
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and proliferation in the otherwise homicidal macrophages. It hinders the phagocytic activity of the macrophages and poses explicit repressive effects on the innate as well as T-cell immune responses (Moore et al., 2001; dos Santos et al., 2016). Sharma et al., ascertained the increased expression of IL-10 at the gene as well as protein level and found its strong co-relation with promotion of the disease burden (Sharma et al., 2017). The success of treatment relies on the erasure of the key cytokine of Th2 response i.e. IL-10 (Chandrasekaran et al., 2017). In the present study the levels of IL-10 were found to reduce drastically post treatment in all groups as compared to the sensitive or resistant strain infected controls. HERERI, SAL and RTN suppressed the disease aggravating IL-10 production in the infected mice on both 7 and 14 p.t.d. Similarly phenylpropanoid dimers isolated from plant, Nectandra leucantha was found to show its antileishmanial efficacy through immunopotentiation. These dimers suppressed the secretion of IL-10 and IL-6 cytokines in macrophages infected with L. donovani (Costa-Silva et al., 2015). In another study mahanine, a carbazole alkaloid was found to control the infection of L. donovani by increasing the levels of IFN-γ and decreasing those of IL-10 and IL-4 (Roy et al., 2017a).

Many studies validate the role of IL-4 in entrusting susceptibility to L. donovani (Murray et al., 2003). In the current study also the high levels of IL-4 in the serum of sensitive or resistant strain infected animals were detected as compared to the treated groups. However the levels were found to decrease in the infected animals after treatment with HERERI, SAL or RTN on both p.t.d. Similar studies conducted on Tinospora cordifolia (100 mg/kg for 15 days) revealed that its administration to L. donovani infected mice protected it from infection by promoting Th1 environment and by declining the levels of IL-4 (Sachdeva et al., 2014). The mRNA expression of IL-10 and IL-4 was found to be downregulated in L. donovani infected BALB/c mice after oral administration of alcoholic fractions of W. somnifera and a purified component, withaferin-A (Chandrasekaran et al., 2017). In the present study SAL and RTN strongly suppressed the secretion of IL-10 and IL-4 in the S- as well as R-strain infected animals. This finding could be correlated with the inhibitory action of SAL and RTN against L. donovani and it also suggests their efficacy to ameliorate the disease pathogenicity caused not only by sensitive but by resistant parasites too.
The therapeutic cure of VL is chiefly dependent upon the establishment of functional immune response that successfully triggers macrophages to generate toxic oxygen and nitrogen species to kill the parasite (Mookerjee Basu et al., 2006). iNOS is a requisite for the synthesis of NO as it catalyses the reaction involving conversion of arginine to NO. The latter is the key agent with microbicidal activity against many parasites including *Leishmania*. NO production is known to restrict not only the growth of parasite but also lead to its death (Bogdan et al., 2000). Further various genes related to the inflammation including TNF-α are controlled by the transcription factor NF-κB (Koutsoni et al., 2014). Therefore in the present study the quantification of the iNOS and NF-κB gene expression was done by RT-PCR. During infection of *L. donovani*, downregulation of expression of number of genes occur which favors the survival of parasite in otherwise hostile macrophages (Buates and Matlashewski, 2001). The expression of genes of transcription factors like NF-κB and RelB (p65 subunit of NF-κB) which further modulates the expression of genes involved in inflammatory responses get downregulated during VL (Lu, 2000). Moreover recent studies have proved that NF-κB regulates the expression of gene iNOS which stimulates the production of microbicidal NO. Thus inactivation of NF-κB further suppresses the expression of iNOS gene (Orsini et al., 2016). Moreover *L. donovani* survives and grows in the mammalian host by activating hypoxia inducing factor-1α and micro RNA-210 (miR-210). The silencing of miR-210 induces translocation of NF-κB into the nucleus and promotes the transcription of many pro-inflammatory cytokines and thus kills the parasite (Kumar et al., 2018). In the present study also downregulation of Nuclear factor-κB and thus inducible NOS genes was found in the animals after infection with either S- or R-strain infected animals. However the expression of these genes was found to enhance in sensitive or resistant strain infected animals after SAL or RTN treatment. These active components upregulated the expression of NF-κB and iNOS genes which further lead to increase in the production of NO. A number of previous studies have revealed that the generation of NO is indispensable to control VL infection. A study carried by Bhattacharjee et al., 2008 demonstrated that quassin increased the expression of mRNA of iNOS in *L. donovani* infected macrophages. Further the high levels of NO and potent immune system was
found to be generated after the treatment of infected macrophages with quassin. In another study the therapeutic potential of ursolic acid (UA), isolated from *Baccharis uncinella* was demonstrated in *L. (L.) infantum* infected hamsters. It was found that UA (2mg/kg for 15 days) increased the mRNA levels of IFN-γ and iNOS and thus controlled the infection (Jesus *et al.*, 2017).

Similarly it was demonstrated that 18beta-glycyrrhetinic acid (GRA) depicted its inhibitory activity against *Leishmania* infection in the *in vitro* and *in vivo* system by up-regulating the expression of NF-κB through the p38 MAPK pathway. It decreased the parasite burden in the spleen by phosphorylating and degrading the IκBα (Ukil *et al.*, 2011). GRA was also found to increase the NO production in macrophages via activating iNOS through activation of NF-κB (Jeong and Kim, 2002). The mechanism of toxicity of alkaloid fraction of *Nuphar lutea* against *L. major* was mediated by increased expression of NF-κB, iNOS genes and production of NO via L-arginine-NO pathway (Ozer *et al.*, 2010). The alleviation of infection after treatment with SAL and RTN in S- or R-strain infected mice can be connected to their ability to modulate the transcriptional capacity of the host cell.

Further in the present study the levels of NO and ROS were analyzed in the spleen of different groups of animals by griess reagent and H2DCFDA dye respectively. Previous studies suggest that the activation of arginase of the parasite by insulin-like growth factor-I influence the host’s macrophages and guides them to check the production of NO via restricting the expression of iNOS (Goto and Prianti, 2009). Further ROS is produced in pathogen infected cells to combat the infection. Infact the mode of action of many antiprotozoan drugs is based on generation of these oxidative species (Fonseca-Silva *et al.*, 2011). Like NO the parasite evades ROS too by an elaborate mechanism which limits the production of superoxide during uptake of parasites by macrophages (Moradin and Descoteaux, 2012). Similar observation was obtained in the present study as the concentrations of these oxidative molecules were found to be down-regulated in the S- or R-strain infected animals. However SAL and RTN successfully enhanced the secretion of ROS and NO in the spleen of either S- or R-strain infected animals. The results are consistent with previous study which demonstrated that RTN improved the endothelial potential by elaborating NO in the
cultured human umbilical vein endothelial cells (HUVEC). RTN increased expression of the endothelial nitric oxide synthase enzyme in HUVEC by 2.1-fold than the control untreated cells which in turn enhanced the NO to 4.095±0.203 μM as compared to only 1.605±0.08 μM in the control (Ugusman et al., 2014). In another study protective efficacy of RTN was investigated against indomethacin induced gastropathy in rats. Pre-treatment with RTN before administration of indomethacin increased the levels of total nitrite/nitrate to 310±13 as compared to only 210±22 nmol/g tissue in indomethacin treated group. This property of RTN was ascribed to its ability to activate the expression of cNOS in the mucosa of stomach (Abdel-Raheem, 2010).

There are many prior studies supporting the pivotal role of ROS and NO in controlling VL infection. The ethanolic extract and the n-butanol fraction of Tinospora sinensis (500 mg/kg b.wt.) exhibited antileishmanial activity in hamsters by increasing appreciable levels of ROS and NO (Singh et al., 2008). In the study of Kyriazis et al., Oleuropein, a biophenol was found to minimize the parasite load via generating the favorable Th1 immune response which led to ROS and NO mediated death of the parasite in the infected BALB/c mice (Kyriazis et al., 2016). Many other natural products like asiaticoside (Bhaumik et al., 2011) and fucoidan (Kar et al., 2011) have been found to demonstrate the antileishmanial efficacy by increasing the levels of these reactive oxygen species. In the study of Shakya et al., 2011 immunomodulator, picroliv was administered to L. donovani infected hamsters in combination with fluconazole and miltefosine. The combination increased the protective efficacy from 77 to 88%. The incorporation of picroliv further enhanced the levels of ROS, NO and strengthened the immune system in the favor of host. The active compounds, lignin and niranthin isolated from Phyllanthus amarus showed profound efficacy against antimony sensitive and resistant L. donovani. The therapeutic effect was achieved by poisoning the topoisomerase I of parasite and by immunostimulation. The strong mounting of Th1 immune status and generation of ROS and NO was accounted after the treatment (Chowdhury et al., 2012). Similarly the treatment of clerodane diterpene controlled the L. donovani infection by elevating the generation of intracellular ROS and depolarization of the mitochondria (Kathuria
The sesquiterpenes, namely mexicanin I, dehydroleucodine and psilostachyin exhibited antiproliferative activity against *L. mexicana mexicana* by inducing oxidative stress in terms of ROS (Barrera *et al*., 2013).

The resistant parasites can thrive better than that of sensitive strains in the presence of oxidative molecules like NO and ROS by increasing the levels of thiols (Verma *et al*., 2017). Moreover resistant parasites over-express γ-glutamylcysteine synthetase gene and thus can compensate the oxidative environment by generating high levels of glutathione. The latter induce increased antioxidant ability to parasite (Carter *et al*., 2006). In the present study although the administration of SSG increased NO and ROS production in sensitive-strain infected animals but it failed to generate NO and ROS molecules in R-strain infected animals. However it was noticed that SAL and RTN participated well in generating high levels of reactive oxygen and nitrogen species in not only sensitive but also resistant parasite infected animals. This implies that these active components can counteract even the resistant behavior of parasite.

The available standard drugs are facing criticism due to their adverse side-effects on important organs like liver, kidney and heart. Over the years the drug discovery related to leishmaniasis mainly focusses on the genesis of not only strong but equally safe therapeutics (Chouhan *et al*., 2015). In most of the reports, the impaired levels of markers of hepatic and renal functions in serum have been associated with disrupted architecture of liver and kidney and disfigurement of integrity of plasma membrane of hepatic and renal cells (Dkhil *et al*., 2014; Kwo *et al*., 2017). Therefore in the present study any kind of injury or physiological change of kidney and liver was assessed biochemically and histologically.

Derangement of liver functions is a common presenting feature of fatal VL. The extent of functional loss of liver is often correlated with the expanse of the disease and to the outcome of treatment (Mathur *et al*., 2008). In the current study impairment of the functions of liver was evidenced in the animals after infection with either strain on both 7 and 14 p.t.d. The infected animals showed higher levels of SGPT, SGOT, LDH, ALP and ACP in the sera. Similarly the treatment groups administered with SSG showed toxicity of liver as depicted by the high levels of these
enzymes in the sera on both 7 and 14 post treatment days. The activity of these enzymes is found to increase only after the dysfunctioning of liver (Limdi and Hyde, 2003). The previous study of Wise et al., 2012 also found disturbances in the levels of liver enzymes after parenteral administration of SSG. However none of the functional biochemicals of liver were found to be altered after treatment with HERERI, SAL and RTN in either sensitive or resistant study groups. The activity of tested liver enzymes was found to be within the normal range on 7 as well as 14 p.t.d. after treatment with either HERERI or the active components. Many past studies support the hepatoprotective efficacy of R. imbricata which has been ascribed to the enormous quantity of the active components present in it especially phenols. The plant protected the liver of rats from the detrimental consequences of paracetamol which could be discerned in terms of deranged biomarkers of the liver. However plant extract at the dosage of 400 mg/kg secured the liver from paracetamol and brought the titres of SGOT, SGPT and ALP within the normal range (Senthilkumar et al., 2014).

Similarly earlier studies substantiate the present data regarding SAL being hepatoprotective in nature. Pretreatment of SAL attenuated the acute effects of toxins, D-galactosamine and lipopolysaccharide via refurbishing the increased levels of AST and ALT in the serum (Wu et al., 2009). Similar results were found with SAL pretreatment in the case of ischemia/reperfusion injury (Cai et al., 2017). Likewise various previous studies have reported the hepatoprotective activity of RTN. It was found to revert the alterations produced by the acetaminophen, paracetamol and CCl4 on the sero-biochemicals of the liver. Pretreatment of RTN nullified the toxic effect in case of administration of paracetamol and CCl4. RTN (20 mg/kg) restored the levels of enzymes of liver viz. ALT, AST and bilirubin in the serum to normal levels and prevented the mortality of the rats (Janbaz et al., 2002; Reddy et al., 2017)

An ideal therapeutic agent should be free from any toxic effects. Thus in the present study the possible side-effects of HERERI and its bioactive components was checked against kidney also. The levels of biomarkers of kidney like urea, BUN, creatinine and uric acid were monitored in the sera of different animals. The implication of kidney in VL is persistent and waste of kidney functions correspond to the chronicity of VL and increased fatality (Daher et al., 2011). The indisposition of
glomerulus during VL is a consequence of polyclonal B-cell activation which disrupts the host immune response (Costa et al., 2010). Different mediators of defense system of the host including NK cells operate via secreting number of cytokines and inflammation causing molecules which results in glomerulopathy and thus reduces the performance of kidneys (Prianti et al., 2007). The present study encountered increased abnormal levels of biomarkers of kidney in the animals after infection with either strain of L. donovani on 7 as well as 14 p.t.d. The present study also documented the nephrotoxicity of AmB as the administration of AmB in infected animals resulted in the derangement of these molecules on 7 as well as 14 p.t.d. depicting the functional loss of activity of kidney. However administration of HERERI, SAL and RTN declined the levels of functional serum biomarkers of kidney within the normal range in infected animals of either strain on both p.t.d.

Heterogenous work has been done to elucidate the protective effect of SAL on various parameters of kidney. For instance, SAL showed the protective effect on the podocytes of mice and shielded them from the apoptotic death caused due to high glucose via promoting HO-1 expression (Lu et al., 2017). Similar defensive effect of SAL was noted on glomerular mesangial cells of rat against high glucose. SAL activated TXNIP-NLRP3 inflammasome pathway and alleviated stress of high glucose on these cells (Wang et al., 2017). SAL reduced albuminuria in diabetic db/db mice model by preventing the transcytosis of albumin across the epithelial cells of glomerulus and by blocking phosphorylation of Cav-1 (Wu et al., 2015). The renal protective effect of RTN has been demonstrated in the previous studies also. Action of co-treatment of RTN with the acute toxicants, cadmium and ethanol was observed in the male Wistar rats as compared to the only cadmium and ethanol administered rats. It was contemplated that rats treated with cadmium and ethanol in the presence of RTN showed normal levels of uric acid i.e. 1.77±0.35 mmol/L as compared to the 3.23±0.55 mmol/L in the serum of rats treated with cadmium and ethanol in the absence of RTN (Abarikwu et al., 2017).

Further to inspect the injurious effects of plant and its bioactive components particularly on the structure of liver, spleen and kidney the histological studies using H and E stains were performed.
During VL liver sustains major architectural disarray involving hepatic and kupffer cells, sinusoids, portal tracts and other blood vessels of liver. Hypertrophy and hyperplasia of the resident macrophages of liver, degeneration of hepatocytes and fibrosis is a common event in VL (Prakash et al., 2006; Khadem et al., 2016). In the present study infiltration of kupffer cells was recorded in the liver of only infected mice. Similar manifestation was documented in the infected mice after SSG treatment which delineated the toxic effects of SSG on the liver. The study by Kato et al., 2014 also suggested the impairment of function of liver by SSG. The hepatocytes depicted various histological changes alongwith many swollen and apoptotic cells after SSG treatment. However plant and both bioactive components were devoid of any toxic retort on liver as evinced by the normal histology of post treatment in sensitive and resistant groups.

The data of present study was in accordance with the former studies where R. imbricata rescinded the toxic effect of paracetamol on liver by abrogating the fibrosis and inflammation of portal tract (Senthilkumar et al., 2014). In many other previous studies hepatoprotection of SAL has been confirmed by histological studies. SAL protected hepatocytes from the apoptotic death by reversing fibrosis of liver cells and decreasing the necrotic region via inducing GSK-3β/Nrf2-dependent response in situations of reperfusion insult or attack of D-galactosamine and lipopolysaccharide on the liver (Guo et al., 2013; Cai et al., 2017). In respect to RTN too analogous results have been found where the RTN regenerated the altered and damaged structure of liver by stabilizing the cell membrane of the hepatocytes. RTN (20 mg/kg) improved the various changes caused by toxic acetaminophen like coagulative necrosis, sinusoidal dilatation and infiltration of mononuclear cells (Reddy et al., 2017). The acquisition of normal histology of liver in HERERI, SAL or RTN treated infected groups demonstrated the protective efficacy of the plant extract and its active components.

The aftermath of many standard antileishmanials including AmB constitute severe kidney manifestations (Sundar and Chatterjee, 2006). The pathogenesis of kidney generated during treatment puts a deleterious effect on the course of disease. Thus in the present study the effect of plant extract, active compounds and the
reference drugs was checked on the architecture of kidney by histological analysis. According to the previous studies the abnormalities of glomerulus of nephrons and interstitial infiltration of mononuclear cells are the frequently observed patterns during kala-azar nephropathy (Kumar et al., 2004). In the present study too infiltrations of lymphocytes were noticed in the untreated infected animals. The pathological findings of kidney in the infected animals represented interstitial nephritis which may be due to the presence of elements of parasite which poses deleterious effect on the course of infection. In the present study the infected animals administered with AmB showed renal insufficiency in terms that these animals revealed protein casts in tubules together with mild damaged tubules. In prior studies too the treatment of AmB has been found to be associated with the acute kidney injury, acidosis of renal tubules, diabetes insipidus and wasting of potassium (Goldman and Koren, 2004; Olliaro et al., 2005). AmB results in vasoconstriction and reduces the filtration rate (Deray, 2002). Moreover it is poorly selective against the cholesterol and ergosterol of the cell membrane and thus causes renal toxicity (Varlam et al., 2001). However in present study HERERI, SAL or RTN and SSG treated infected animals did not show any treatment-related harm to kidney. The glomeruli and tubules were observed to be normal in these infected treated groups. The previous studies support the renal protective performance of plant extract and active compounds. For instance, different species of *Rhodiola* have been found to possess protective qualities against renal diseases. *Rhodiola* rescued the kidney of rats from the damage induced by unilateral ureter obstruction by securing its normal histology (Tong et al., 2010; Uyeturk et al., 2013). Former studies have also depicted the role of RTN in maintaining or repairing the architecture of kidney. Cadmium plus ethanol treatment of rats resulted in severe congestion of cortex region of kidney as well as damage of glomerulus. However administration of RTN along with cadmium and ethanol sustained the normal renal morphology with no evident lesions (Abarikwu et al., 2017). The findings of the current study depicted no side-effects of HERERI or active compounds on kidney.

Like liver, spleen is another organ crucial during VL. It participates in the immune surveillance against pathogens circulating in the blood. The morphology and
the structural organization of spleen signifies the progress of disease (Carrión et al., 2006) and thus in the present study the histological studies of spleen of different groups of animals were conducted. In VL, vanishing of marginal zone separating red and white pulp occur along with increase in the size of spleen. In addition to the atrophy of white pulp the disappearance of lymphoid follicles is common in severe form of VL (d'El-Rei Hermida et al., 2018). In the present study the infected animals of either strain showed disorganized spleen structure. The light microscopic studies of H and E stained slides revealed blurry marginal zone between the red and white pulp in the infected animals. The pathology of spleen in the infected animals indicated the parasite infection. However no signs of damaged spleen were observed in the infected animals after treatment with either plant extract or bioactive components. The infected animals treated with either standard drug, SSG or AmB depicted no side-effects on spleen. A clear demarcation between the red and white pulp was evident in the spleen of all the treated groups. In the treated animals boundaries separating the two pulp regions i.e. the marginal zone was clearly visible. The normal histology of spleen observed in the HERERI, SAL or RTN treated infected groups distinctly certified that the plant extract and the active compounds have no side-effects on spleen. Moreover the fact that normal structure and function of spleen was acquired after HERERI, SAL or RTN treatment supported their ability in controlling the *L. donovani* infection.

The results obtained in the present study are encouraging in terms of promising effect of HERERI, SAL and RTN against *L. donovani*. The plant extract and the active compounds controlled the parasite load by establishing an effective Th1 response concomitant to the increase in the levels of microbicidal molecules, ROS and NO. The antiparasitidal effect of SAL and RTN was found to be functional against not only sensitive but also against the resistant strain. This highlighted the relevancy of SAL and RTN in counteracting against the emergence of resistance by parasite. From the findings it can be deduced that the active compounds possess the ability to overcome the limitations related to the ongoing conventional antileishmanials and thus can contribute as a leishmanicidal agent.