Protection against experimental visceral leishmaniasis by immunostimulation with herbal drugs derived from *Withania somnifera* and *Asparagus racemosus*

Sukhbir Kaur, Kalpana Chauhan and Heena Sachdeva

Department of Zoology, Panjab University, Chandigarh-160014, India

Visceral leishmaniasis (VL) is a vector-borne parasitic disease targeting tissue macrophages. It is among the most neglected infectious diseases. As available therapeutics for treatment of this disease have many side effects, there is a need for safer alternatives. One of the immunopathological consequences of active visceral leishmaniasis is suppression of protective T-helper (Th)-1 cells and induction of disease-promoting Th-2 cells, and thus the treatment of VL relies on immunomodulation.

In the current study, herbal drugs derived as whole-plant extracts of *Asparagus racemosus* and *Withania somnifera* were used to treat *Leishmania donovani*-infected BALB/c mice. Keeping the scenario of immunosuppression during VL in mind, the potential of these drugs in the restoration of murine Th-1-type protective immune responses was evaluated. To investigate the propensity of these drugs to treat VL, liver parasite load, delayed-type hypersensitivity responses and parasite-specific immunoglobulin levels were studied. Various biochemical and haematological tests were also carried out. A positive-control group used the standard drug treatment of sodium stibogluconate. Treatment of infected mice with *A. racemosus* and *W. somnifera* in combination at the higher dose of 200 mg (kg body weight)$^{-1}$ not only resulted in a successful reduction in parasite load but also generated protective Th1-type immune responses with normalization of biochemical and haematological parameters, suggesting their potential as potent anti-leishmanial agents.

INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar is the most severe form of leishmaniasis and is responsible for most *Leishmania*-associated deaths. VL represents a serious public health problem that affects many countries (Paletta-Silva & Meyer-Fernandes, 2012). There are an estimated 500,000 new cases per year of VL globally (Moore & Lockwood, 2010). The annual estimate for the incidence and prevalence of VL cases worldwide is 0.5 million and 2.5 million, respectively. Of these, 90% of the confirmed cases occur in India, Nepal, Bangladesh and Sudan. In India, the disease is a serious problem in Bihar, West Bengal, Jharkhand and eastern Uttar Pradesh (Bora, 1999).

In the absence of vaccines, pentavalent antimonic drugs like sodium stibogluconate (SSG) and meglumine antimoniate have remained the first-line therapy for VL for over half a century, and are still in use in many parts of the world.

However, the prolonged treatment requiring parenteral administration, toxicity and the emergence of significant resistance are all factors limiting the usefulness of this drug (Croft & Coombs, 2003). Although a few other options are available such as amphotericin B, miltefosine, paromomycin and lipid-conjugated formulations of amphotericin B, they also suffer from one or more limitations (Maltezou, 2010). Therefore, there is a need to develop newer drug therapies that are more efficacious and safer. There are many immunosuppressive or immune-evasion mechanisms contributing to the pathogenesis of VL. *Leishmania* infection is classically associated with a depression of T-helper type 1 (Th1) cells and preferential expansion of Th2 cells (Awasthi et al., 2004). Therefore, recovery from leishmaniasis is generally affected by upregulation of cellular immune responses capable of activating host macrophages and involves activation of Th1 cells. Plant extracts or plant-derived compounds are likely to provide a valuable source of new medicinal agents, and the urgent need for alternative treatments has led to a programme to screen natural products for potential use in the therapy of leishmaniasis (de Carvalho & Ferreira, 2001).

Medicinal plants are a good source of bioactive agents that are useful against many diseases, including leishmaniasis, and their active components have been shown to be an important...
source of immunomodulators (Ganguly & Prasad, 2011). Plant products have been shown to have an immunoregulatory effect on the host’s immune system. Various plant extracts and herbal feed additives at a specific dose may be helpful in obtaining higher protective antibody titres and more effective cell-mediated immune responses for protection against various bacterial, viral and other diseases (Kanungo & Sahoo, 2011). For instance, crude ethanolic extract of the Indian medicinal plant *Desmodium gangeticum* and its fractions have chemoprophylactic and chemotherapeutic properties against VL (Mishra *et al.*, 2005). Extracts of *Echinacea purpurea* display multiple immunomodulatory activities, comprising stimulation of certain immune functions such as phagocytic activity of macrophages, and show leishmanicidal activity (Soudi *et al.*, 2007). *Withania somnifera*, commonly known as ashwagandha, belongs to the family Solanaceae. It is widely used in the treatment of inflammatory conditions (Gupta & Singh, 2014), rheumatism, different types of tumours, tuberculosis, and conditions of the reproductive system and nervous system, is beneficial to improve vitality and is a good immunomodulator (Kanungo & Sahoo, 2011). Administration of an extract from the powdered root of *W. somnifera* has been found to stimulate immunological activity in BALB/c mice. Treatment with *Withania* root extract (20 mg per dose per animal; intraperitoneal injection) has been found to enhance the total white blood cell (WBC) count and bone-marrow cellularity. When *Withania* extract was used along with the antigen [sheep red blood cells (SRBC)], it enhanced the circulating antibody titres, number of plaque-forming cells in the spleen and phagocytic activity of peritoneal macrophages. These results confirmed the immunomodulatory activity of *W. somnifera* extract (Davis & Kuttan, 2000). *Asparagus racemosus*, commonly known as shatavari, has potent antioxidant (Palanisamy & Manian, 2012), anti-dyspepsia, neuroprotective (Prakash *et al.*, 2013; Kurapati *et al.*, 2013) and anti-tussive effects. It significantly enhances the total WBC count, haemoglobin content and bone-marrow cellularity in animals treated with cyclophosphamide. It enhances the weight of lymphoid organs such as thymus and spleen, which indicates its effect on cellular immune responses. It also affects stem-cell differentiation and overcomes the immunosuppression (Begum & Anuradha, 2011).

The present study was planned to evaluate the immunomodulatory and anti-leishmanial efficacy of two herbal drugs derived from whole-plant extracts of *W. somnifera* and *A. racemosus* in Leishmania donovani-infected BALB/c mice and to compare their efficacy with the standard available anti-leishmanial drug, SSG.

**METHODS**

**Parasite.** The Indian strain of *L. donovani*, MHOM/IN/80/Dd8, originally obtained from the London School of Tropical Hygiene and Medicine, UK, was used for the present study. The promastigotes of this strain were maintained in modified Novy, McNeal and Nicolle’s (NNN) medium with minimal essential medium as overlay.

**Assay of parasite-specific IgG1 and IgG2a isotypes by ELISA.** The serum-specific IgG isotype antibody responses were measured by conventional ELISA. To prepare the antigen, promastigotes were harvested from NNN medium and then washed three times with PBS. The suspension of promastigotes was freeze–thawed (six cycles). The protein concentration was estimated and the sample was stored at −20 °C until further use. A polystyrene micro–ELISA plate was coated with 10 μg crude antigen (promastigote lysate) and incubated at 4 °C overnight. The antigen solution was then decanted and the plate was

**Animals.** Inbred BALB/c mice of either sex (5–6 weeks old) weighing about 20–25 g were obtained from the Institute of Microbial Technology and Central Animal House, Panjab University, Chandigarh, India, and were housed in the experimental wing and fed with water and mouse feed *ad libitum*. Ethical clearance for conducting the experiments was obtained from the Institutional Animal Ethics Committee (IAEC/158-169/ dated-29-08-2011), Panjab University, Chandigarh, India.

**Drugs.** *W. somnifera* and *A. racemosus* were purchased as pure herbs in the form of tablets (of whole plant) from the Himalaya Drugs company, Bangalore, India. These were dissolved in distilled water to get the required concentration as mg (kg body weight of mice)^−1^ (hereafter mg kg^−1^). SSG was purchased from Wellcome Research Laboratories, UK, and dissolved in double-distilled water in a water bath at 72 °C to obtain the required concentration of 40 mg kg^−1^. It was injected intraperitoneally into mice daily for 5 days (Sodhi et al., 1992).

**Experimental design.** The following groups of mice were used: group 1, normal control given PBS only; group 2, infected control comprising inbred BALB/c mice injected intracardially with 10^7^ promastigotes of *L. donovani* (these mice were left for 1 month for the progressive development of the disease); group 3, positive control comprising infected mice treated with SSG at a dose of 40 mg kg^−1^; group 4, infected mice administered *W. somnifera* alone daily at a dose of 200 mg kg^−1^ orally for 7 days; group 5, infected animals administered *A. racemosus* alone daily at a dose of 200 mg kg^−1^ orally for 7 days; group 6, infected animals treated daily with a low-dose combination of *A. racemosus* + *W. somnifera* (100 mg kg^−1^ each) orally for 7 days; group 7, infected animals treated daily with a high-dose combination of *A. racemosus* + *W. somnifera* (200 mg kg^−1^ each) orally for 7 days.

Eighteen mice were taken at the start in each group and six were sacrificed on days 37, 44 and 52 post-infection (p.i.) or on days 0, 7 and 15 post-treatment (p.t.) for the analysis of various parameters.

**Assessment of infection.** Mice from each group were sacrificed on days 37, 44 and 52 p.i. or on days 0, 7 and 15 p.t. The hepatic parasite load in all groups of BALB/c mice was assessed on Giemsa-stained blood smears. For the estimation of hepatic parasite load, the liver was removed aseptically from each group of mice and homogenized in sterile RPMI-1640 medium. The homogenate was serially diluted from 10^−1^ to 10^−6^ and 50 μl was used for each dilution. The dilutions were mounted, stained with Giemsa stain, and examined under light microscope for the presence of parasites. The number of parasites per high magnification field was counted, and the average number of parasites per liver cell was calculated from 10–20 fields.

**Delayed-type hypersensitivity (DTH) responses.** Promastigotes in the stationary phase of growth were harvested from NNN medium and washed with PBS and pelleted three times. The final pellet was resuspended in 5 ml 0.5% (w/v) phenol in sterile PBS to kill the promastigotes. The phenol was then removed by three washes with PBS and the concentration was adjusted to 2 x 10^9^ parasites ml^−1^. All groups of mice were challenged in the right foot pad with a subcutaneous injection of 40 μl leishmanin 48 h before the sacrifice, which serves as an effector phase. The control left paw received an equal volume of PBS only. After 48 h, the thickness of the right and left foot pads was measured using a pair of Vernier callipers. The percentage increase in the thickness of the right foot pad compared with the left foot pad was calculated (Nagil et al., 2009) using formula: [(thickness of right foot pad – thickness of left foot pad)/ thickness of left foot pad] x 100.

**Experiment.** The following groups of mice were used: group 1, normal control given PBS only; group 2, infected control comprising inbred BALB/c mice injected intracardially with 10^7^ promastigotes of *L. donovani* (these mice were left for 1 month for the progressive development of the disease); group 3, positive control comprising infected mice treated with SSG at a dose of 40 mg kg^−1^; group 4, infected mice administered *W. somnifera* alone daily at a dose of 200 mg kg^−1^ orally for 7 days; group 5, infected animals administered *A. racemosus* alone daily at a dose of 200 mg kg^−1^ orally for 7 days; group 6, infected animals treated daily with a low-dose combination of *A. racemosus* + *W. somnifera* (100 mg kg^−1^ each) orally for 7 days; group 7, infected animals treated daily with a high-dose combination of *A. racemosus* + *W. somnifera* (200 mg kg^−1^ each) orally for 7 days.
washed with washing buffer. Next, 100 μl 4% BSA was added to the wells and the plate was incubated at 37°C for 1 h, followed by further washings. Infected mouse serum samples (50 μl) diluted 1:4 in PBS were added and incubated at 37°C for 1 h, and the washing procedure was repeated. One hundred microlitres of the anti-mouse secondary antibody conjugated to horseradish peroxidase was added at a dilution of 1:8400 for IgG1 (AbD Serotec) and 1:2000 for IgG2a (AbD Serotec) antibody conjugated to horseradish peroxidase. Absorbance was read on an ELISA plate reader at 450 nm (Nagill et al., 2009).

Liver function tests. Serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), bilirubin and alkaline phosphatase (ALP) were estimated in serum samples of all groups of mice using commercially available kits.

SGPT and SGOT estimation was carried out colorimetrically using biochemical kits following the manufacturer’s instructions (Kinetik Koncepts), and results were expressed in U l\(^{-1}\).

ALP estimation was carried out colorimetrically using a biochemical kit following the manufacturer’s instructions (Span Diagnostics), and results were expressed as King-Armstrong units (KAU) in plasma.

Bilirubin estimation was carried out colorimetrically using a biochemical kit supplied by Rekon Diagnostics, and results were expressed in mg dl\(^{-1}\).

Kidney function tests. Blood urea, blood urea nitrogen (BUN) and blood creatinine levels were estimated in serum samples of all groups of mice using commercially available kits following the manufacturer’s instructions (Kinetik Koncepts), and results were expressed in mg (dl plasma)\(^{-1}\).

Evaluation of haematological parameters

Estimation of haemoglobin (Hb). The blood of mice from all groups was collected and Hb estimation was carried out using a Sahli haemometer. HCl (0.1 M) was added to the haemoglobinometer tube up to the 2 ml mark with a dropper. The blood was sucked into the pipette up to 0.2 ml mark from the cut end of the tail of the mice. The blood was transferred into 0.1 M HCl in the tube. The blood and 0.1 M HCl were mixed with a stirrer and then left for 5–10 min. Distilled water was then gently added drop by drop into the acid/blood mixture. When the colour of the mixture matched the colour of the standard tube, this was taken as the end point and gave the value of haemoglobin in g% (Wintrobe, 1975).

Estimation of total leukocyte count (TLC). Estimation of TLC was carried out with a haemocytometer. Blood was diluted with diluting fluid in a ratio of 1:20 in a glass pipette. The contents were mixed for at least 1 min and 10 μl of this mixture was placed in a Neubauer counting chamber and leukocytes were counted in the four corner areas containing 16 squares each. TLC was calculated using the following formula: (number of leukocytes counted × dilution factor)/(area counted × depth of fluid) × 10\(^6\).

Statistical analysis. Student’s t-test was performed to calculate the P value using Graphpad Prism 5.0 software.

RESULTS

Parasite load

In the infected animals (group 2), the parasite load increased from 3989 ± 32.14 (mean ± SD) on day 37 p.i. to 4998 ± 71.22 on day 52 p.i. However, in infected animals treated with W. somnifera (200 mg kg\(^{-1}\); group 4), the parasite load was found to be significantly decreased (P<0.0001) to 3713 ± 83.79, 3395 ± 55.86 and 2945 ± 47.08, and in infected animals treated with A. racemosus (200 mg kg\(^{-1}\); group 5), the parasite load was 3633 ± 110.61, 3137 ± 74.30 and 2764 ± 139.42, on days 0, 7 and 15 p.t., respectively, which also was significantly (P<0.0001) less compared with infected controls (Fig. 1). Similarly, the parasite load in infected animals treated with W. somnifera in combination with A. racemosus at the dose of 100 mg kg\(^{-1}\) each (group 6) was found to be 3060 ± 92.73, 2311 ± 65.93 and 1665 ± 81.24, whilst in infected animals treated with W. somnifera in combination with A. racemosus at the dose of 200 mg kg\(^{-1}\) each (group 7), the parasite load was 2122 ± 86.30, 1527 ± 84.29 and 925 ± 32.06, on days 0, 7 and 15 p.t., respectively, which was significantly lower (P<0.0001) compared with the infected controls (Fig. 1). However, the parasite burden in the infected animals treated with SSG (group 3) was found to be significantly (P<0.0001) decreased to 1015 ± 28.58, 858.33 ± 43.08 and 612.67 ± 100.65 compared with the infected animals treated with both pure herbs at the dose of 200 mg kg\(^{-1}\) each on the same days p.t., respectively (Fig. 1).

DTH responses

Infected animals treated with W. somnifera and A. racemosus alone at the dose of 200 mg kg\(^{-1}\) each (group 7) and with the low-dose combination of 100 mg kg\(^{-1}\) (group 6) both exhibited increased footpad thickness from 0 to 7 days and from 7 to 15 days p.t. when measured after 48 h of leishmanin challenge, suggesting that they could combat the immunosuppression, as DTH was found to depress the response only in the infected animals (Fig. 2). Our results also revealed that a higher dose of W. somnifera in combination with A. racemosus (200 mg kg\(^{-1}\) each) generated the most significant enhancement in the DTH response with the increase in days p.t. compared with infected controls (group 2) and the low-dose combination of herbal drugs (100 mg kg\(^{-1}\) each) (Fig. 2). However, in infected animals treated with SSG (group 3), the DTH response was found to increase significantly (P<0.05) compared with the animals treated with the herbal drugs in combination at a higher dose (Fig. 2).

Detection of parasite-specific IgG isotype levels

The status of parasite-specific IgG isotypes IgG1 and IgG2a in the serum samples of all the groups of mice was studied by ELISA (Figs 3 and 4). L. donovani-infected BALB/c mice had high IgG1 antibody titres and reduced IgG2a levels compared with normal controls (group 1), which indicated the suppression of protective Th1 responses in this group of animals. However, this pattern was reversed upon disease resolution after treatment with the herbal drugs W. somnifera and A. racemosus alone (200 mg kg\(^{-1}\) each; groups 4 and 5) and with a low-dose (100 mg kg\(^{-1}\) each;
group 6) or high-dose (200 mg kg\(^{-1}\) each; group 7) combination. The maximum decrease in disease-promoting IgG1 and the maximum increase in disease-resolving IgG2a antibody was found in infected animals treated with \textit{A. racemosus} and \textit{W. somnifera} at the high dose of 200 mg kg\(^{-1}\) each from 0 to 15 days p.t., thus indicating the generation of a protective Th1-type immune response. However, in infected animals treated with SSG (group 3),

\[ \text{Parasite load (LDU) in the various groups of animals. Results are expressed as means \pm SD of six animals.} \]

\*P<0.0001 for group 2 vs group 4/group 5/group 6/group 7; \(tP<0.0001\) for group 6 vs group 7; \(tP<0.0001\) for group 3 vs group 7.

\[ \text{DTH responses in the various groups of animals. Results are expressed as means \pm SD of six animals.} \]

\*P<0.0001 for group 1 vs group 2; \(tP<0.0001\), \(tP<0.05\) and \(tP>0.05\) (not significant) for group 2 vs group 4/group 5/group 6/group 7; \(tP<0.0001\) for group 6 vs group 7; \|P<0.05\) for group 3 vs group 7.
the decrease in IgG1 level and increase in IgG2a level was lower when compared with the animals treated with both herbal drugs in combination at the higher dose (200 mg kg$^{-1}$ each).

**Liver function tests**

In the infected animals, the activity of enzymes SGOT and SGPT and concentration of bilirubin were found to be increased, whilst the activity of ALP decreased from 0 to

---

**Fig. 3.** IgG1 levels in the various groups of animals at serial twofold dilutions. Results are expressed as means ± sd of six animals. *P<0.0001 for group 1 vs group 2; †P<0.0001 and ‡P>0.05 (not significant) for group 2 vs group 4/group 5/group 6/group 7; §P<0.0001 for group 6 vs group 7; †P<0.0001 for group 3 vs group 7.

**Fig. 4.** IgG2a levels in the various groups of animals at serial twofold dilutions. Results are expressed as means ± sd of six animals. *P<0.0001 for group 1 vs group 2; †P<0.0001 for group 2 vs group 4/group 5/group 6/group 7; ‡P<0.05 (not significant) for group 2 vs group 4/group 5/group 6/group 7; §P<0.0001 for group 6 vs group 7; †P<0.0001 for group 3 vs group 7.
15 days post-infection (p.i.). However, treatment of infected animals with *A. racemosus* and *W. somnifera* alone at a dose of 200 mg kg\(^{-1}\) each (groups 4 and 5) and with a low-dose (100 mg kg\(^{-1}\) each; group 6) or high-dose (200 mg kg\(^{-1}\) each; group 7) combination prevented toxicity, as the levels of SGOT, SGPT, ALP and bilirubin were within the normal range on all days post-treatment (p.t.). However, the levels of SGOT and SGPT in the infected plus SSG-treated animals (group 3) were found to be increased significantly \((P<0.0001)\) compared with infected animals treated with both herbal drugs at the higher dose (200 mg kg\(^{-1}\) each) (Table 1).

**Kidney function tests**

The concentration of serum creatinine, blood urea and BUN was found to be increased in the infected animals from 0 to 15 days p.i. However, their concentration was found to decrease and was within the normal range in all the groups of infected mice treated with *A. racemosus* and *W. somnifera* alone at the dose of 200 mg kg\(^{-1}\) each (groups 4 and 5) and with a low-dose (100 mg kg\(^{-1}\) each; group 6) or high-dose (200 mg kg\(^{-1}\) each; group 7) combination. However, in the group of infected mice treated with SSG (group 3), serum creatinine levels were found to increase significantly \((P<0.0001)\) compared with infected animals treated with both herbal drugs at the higher dose (200 mg kg\(^{-1}\) each) (Table 2).

**Haematological studies**

In the present study, TLC was found to be increased in the infected animals from 0 to 15 days p.i. However, infected mice treated with *A. racemosus* and *W. somnifera* alone at the dose of 200 mg kg\(^{-1}\) each (groups 4 and 5) and with a low-dose (100 mg kg\(^{-1}\) each; group 6) or high-dose (200 mg kg\(^{-1}\) each; group 7) combination brought about the normal level of TLC. Normal levels of TLC were also found in the infected plus SSG-treated animals (group 3). The haemoglobin concentration was found to be increased in infected mice treated with *A. racemosus* and *W. somnifera* alone at a dose of 200 mg kg\(^{-1}\) each and with a low-dose (100 mg kg\(^{-1}\) each) or high-dose (200 mg kg\(^{-1}\) each) combination compared with the low Hb concentration in infected controls (group 2) where it decreased from 0 to 15 days p.i. A normal Hb concentration was found in infected animals treated with SSG (Table 3).

**DISCUSSION**

In this study, we evaluated a new therapeutic approach with two herbal drugs, *A. racemosus* and *W. somnifera*, against *L. donovani*-infected BALB/c mice. Immunomodulation is a process that can alter the immune system of an organism by interfering with its functions. During leishmaniasis, the immune system becomes severely downregulated, so the present study focused on the upregulation of the immune system, and for this, these plants were selected for the study as they have well-known immunostimulatory properties. The latter property was exploited to upregulate the immune response of *L. donovani*-infected mice, which ultimately resulted in a reduction in parasite load, as seen in the impression smears of liver. LDU was found to be decreased in all the infected animals treated with the herbal drugs. Moreover, the maximum reduction in the parasite load was found when the infected animals were treated with combination of *A. racemosus* and *W. somnifera* at the higher dose of 200 mg kg\(^{-1}\) each. However, the reduction in parasite load in infected animals treated with SSG was greater compared with infected mice treated with the combination of herbal drugs at the higher dose of 200 mg kg\(^{-1}\).

The anti-leishmanial efficacy of *A. racemosus* may be because of the potent anti-leishmanial molecule, racemose A, present in its fruit. It exerts its anti-leishmanial effect through the induction of programmed cell death mediated by loss of plasma membrane integrity and loss of mitochondrial membrane potential culminating in cell-cycle arrest (Dutta *et al.*, 2007). Previous studies have also suggested that the possible mode of parasite killing by *W. somnifera* is due to the presence of withaferin A (steroidal lactone) and withanolide A. It has been proposed that withaferin A may be responsible for causing apoptosis in leishmanial cells by inhibiting protein kinase C, a central protein in generating apoptosis (Sen *et al.*, 2007).

Disease severity in *L. donovani*-infected BALB/c mice was found to be associated with hampered DTH responses and IgG2a levels. This suggests the importance of T-cell-mediated protective immune responses in the treatment of VL (Basu *et al.*, 2005). Thus, we tested immunological outcomes in all the groups of infected mice treated with *W. somnifera* and *A. racemosus* alone at a dose of 200 mg kg\(^{-1}\) each and with a low-dose (100 mg kg\(^{-1}\) each) and high-dose (200 mg kg\(^{-1}\) each) combination. It was observed that, although DTH was found to be increased in all the herbal drug-treated infected animals, the maximum increase was found in those infected animals that were treated with *W. somnifera* in combination with *A. racemosus* at the higher dose of 200 mg kg\(^{-1}\) each. However, the DTH response in the infected animals after treatment with SSG was significantly more compared with the infected animals treated with both herbal drugs at the higher dose of 200 mg kg\(^{-1}\) each. These results are in accordance with a previous study where an ethanolic extract of *W. somnifera* and *A. racemosus* was found to exhibit significant DTH responses to SRBCs in mice (Siddiqui *et al.*, 2012).

IgG2a and IgG1 kinetics indirectly reflect the Th1/Th2 responses and thus can be utilized as a marker for the induction of a Th1- or Th2-type immune response (Siddiqui *et al.*, 2012). All infected animals treated with herbal drugs also showed an elevated level of the serum anti-leishmanial immunoglobulin IgG2a isotype, with the maximum increase in the group of infected animals treated with a combination of both herbal drugs at a higher dose of 200 mg kg\(^{-1}\) each, whereas only infected mice failed to mount an anti-leishmanial IgG2a response and instead
Table 1. Estimation of liver function tests in the various groups of BALB/c mice

<table>
<thead>
<tr>
<th>Animal group</th>
<th>SGOT activity (U l⁻¹)</th>
<th>SGPT activity (U l⁻¹)</th>
<th>ALP (KAU)</th>
<th>Bilirubin (mg dl⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 days p.i./p.t.</td>
<td>7 days p.i./p.t.</td>
<td>15 days p.i./p.t.</td>
<td>0 days p.i./p.t.</td>
</tr>
<tr>
<td>Group 1: normal</td>
<td>39.66 ± 0.46</td>
<td>38.97 ± 0.67</td>
<td>38.59 ± 0.42</td>
<td>31.93 ± 1.43</td>
</tr>
<tr>
<td>Group 2: infected</td>
<td>46.29 ± 0.50*</td>
<td>50.51 ± 0.33*</td>
<td>51.37 ± 0.98*</td>
<td>42.92 ± 0.11*</td>
</tr>
<tr>
<td>Group 3: infected + SSG (40 mg kg⁻¹)</td>
<td>52.79 ± 1.35</td>
<td>46.13 ± 1.33</td>
<td>43.73 ± 1.44</td>
<td>57.07 ± 0.88</td>
</tr>
<tr>
<td>Group 4: infected + W. somnifera (200 mg kg⁻¹)</td>
<td>36.19 ± 0.33†</td>
<td>36.90 ± 0.14†</td>
<td>35.59 ± 0.49†</td>
<td>30.80 ± 1.64†</td>
</tr>
<tr>
<td>Group 5: infected + A. racemosus (200 mg kg⁻¹)</td>
<td>34.99 ± 0.07†</td>
<td>33.74 ± 0.29†</td>
<td>34.64 ± 0.44†</td>
<td>31.60 ± 1.28†</td>
</tr>
<tr>
<td>Group 6: infected + W. somnifera and A. racemosus (100 mg kg⁻¹ each)</td>
<td>32.39 ± 0.75†</td>
<td>33.21 ± 0.99†</td>
<td>33.19 ± 1.60†</td>
<td>28.25 ± 0.31†</td>
</tr>
<tr>
<td>Group 7: infected + W. somnifera and A. racemosus (200 mg kg⁻¹ each)</td>
<td>32.34 ± 0.02††§</td>
<td>34.11 ± 0.17††§</td>
<td>32.97 ± 0.05††§</td>
<td>21.60 ± 1.68†§</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SD of six animals. p.i., Post infection; p.t., post-treatment.

*P < 0.0001 for group 1 vs group 2.
†P < 0.0001 for group 2 vs group 4/group 5/group 6/group 7.
‡P < 0.0001 for group 6 vs group 7.
§P < 0.0001 for group 3 vs group 7.
∥P > 0.05 (not significant) for group 6 each vs group 7.
¶P > 0.05 (not significant) for group 3 vs group 7.
#P < 0.05 for group 3 vs group 7.
## Table 2. Estimation of kidney function tests in the various groups of BALB/c mice

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Blood urea (mg dl⁻¹)</th>
<th>BUN (mg dl⁻¹)</th>
<th>Creatinine (mg dl⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 days p.i./p.t.</td>
<td>7 days p.i./p.t.</td>
<td>15 days p.i./p.t.</td>
</tr>
<tr>
<td>Group 1: normal</td>
<td>27.99 ± 2.00</td>
<td>30.34 ± 0.55</td>
<td>26.97 ± 1.39</td>
</tr>
<tr>
<td>Group 2: infected</td>
<td>25.26 ± 2.00*</td>
<td>55.33 ± 3.77*</td>
<td>73.67 ± 2.56*</td>
</tr>
<tr>
<td>Group 3: infected + SSG (40 mg kg⁻¹)</td>
<td>36.30 ± 0.97</td>
<td>42.57 ± 1.22</td>
<td>37.27 ± 1.65</td>
</tr>
<tr>
<td>Group 4: infected + W. somnifera (200 mg kg⁻¹)</td>
<td>37.32 ± 1.69†</td>
<td>38.93 ± 1.80†</td>
<td>38.16 ± 2.63†</td>
</tr>
<tr>
<td>Group 5: infected + A. racemous (200 mg kg⁻¹)</td>
<td>38.32 ± 2.06†</td>
<td>39.96 ± 1.67†</td>
<td>36.81 ± 2.33†</td>
</tr>
<tr>
<td>Group 6: infected + W. somnifera and A. racemous (100 mg kg⁻¹ each)</td>
<td>32.01 ± 1.36†</td>
<td>38.11 ± 1.29†</td>
<td>37.07 ± 1.07†</td>
</tr>
<tr>
<td>Group 7: infected + W. somnifera and A. racemous (200 mg kg⁻¹ each)</td>
<td>30.50 ± 1.73†‡‡</td>
<td>37.68 ± 2.02‡‡</td>
<td>35.39 ± 4.10‡‡</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SD of six animals. p.i., Post infection; p.t., post-treatment.

*P<0.0001 for group 1 vs group 2.

‡P<0.0001 for group 2 vs group 4/group 5/group 6/group 7.

§P>0.05 (non-significant) for group 6 vs group 7.

‡‡P<0.0001 for group 3 vs group 7.

∀P<0.05 for group 3 vs group 7.

∀∀P<0.0001 for group 3 vs group 7.
Table 3. Estimation of haematological parameters in the various groups of BALB/c mice

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Hb concentration (g dl⁻¹)</th>
<th>TLC (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 days p.i./p.t.</td>
<td>7 days p.i./p.t.</td>
</tr>
<tr>
<td>Group 1: normal</td>
<td>11.00 ± 0.81</td>
<td>10.70 ± 0.980</td>
</tr>
<tr>
<td>Group 2: infected</td>
<td>8.33 ± 0.94*</td>
<td>7.83 ± 0.620*</td>
</tr>
<tr>
<td>Group 3: infected + SSG (40 mg kg⁻¹)</td>
<td>10.93 ± 0.41</td>
<td>11.07 ± 0.41</td>
</tr>
<tr>
<td>Group 4: infected + W. somnifera (200 mg kg⁻¹)</td>
<td>10.43 ± 0.44‡</td>
<td>11.06 ± 0.73‡</td>
</tr>
<tr>
<td>Group 5: infected + A. racemosus (200 mg kg⁻¹)</td>
<td>10.13 ± 0.57‡</td>
<td>11.23 ± 0.55‡</td>
</tr>
<tr>
<td>Group 6: infected + W. somnifera and A. racemosus (100 mg kg⁻¹ each)</td>
<td>11.26 ± 0.45‡</td>
<td>11.80 ± 0.50‡</td>
</tr>
<tr>
<td>Group 7: infected + W. somnifera and A. racemosus (200 mg kg⁻¹ each)</td>
<td>11.77 ± 0.75§</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as means ± SD of six animals. p.i., Post infection; p.t., post-treatment.
*P < 0.05 for group 1 vs group 2.
†P < 0.0001 for group 1 vs group 2.
‡P < 0.0001 for group 2 vs group 4/group 5/group 6/group 7.
§P > 0.05 (non-significant) for group 6 vs group 7.
||P > 0.05 (non-significant) for group 3 vs group 7.
¶P < 0.0001 for group 6 vs group 7.
#P < 0.0001 for group 3 vs group 7.
**P > 0.05 (non-significant) for group 3 vs group 7.
showed an elevated level of IgG1. This indicated that these herbal drugs could combat murine *L. donovani* infection by expanding the response of Th1 cells. However, the increase in levels of IgG2a and decrease in that of IgG1 in the infected plus SSG-treated positive-control group was not as great as that as found in the infected group treated with both herbal drugs at the higher-dose combination (200 mg kg$^{-1}$ each). Our results are in accordance with the reports of Gautam *et al.* (2004) who showed the up-regulation of Th1 and Th2 cytokines after administration of *A. racemosus* aqueous root extract in SRBC-sensitized animals, thus suggesting Th1/Th2 adjuvant activity. Aqueous root extract-treated animals also showed a significant increase in DTH responses, antibody titres and CD3$^+$, CD4$^+$, CD8$^+$ percentages, indicating its effect on T-cell activation (Shimizu *et al.*, 2003). Such modulation by *W. somnifera* was reported previously by Malik *et al.* (2007) who stated that *W. somnifera* aqueous root extract stimulated cell-mediated immunity, IgM, IgG and enhanced proliferation of CD3$^+$, CD4$^+$, CD8$^+$, CD19, IFN-γ, IL-2 and IgG2a in the immune system of SRBC-immunized BALB/c mice. They also suggested that withanolide A from *W. somnifera* appeared to be responsible for the Th1 skewing effect (Gautam *et al.*, 2004). *W. somnifera* ethanolic extract was found to enhance the humoral and cell-mediated immune response on day 7 by 12 and 19.2%, respectively, compared with the standard control of cyclophosphamide, which resulted in an enhancement of 54% humoral immune response and 37.63% cell-mediated immune response (Malik *et al.*, 2007).

Both herbal drugs, *A. racemosus* and *W. somnifera*, were also used in this experimental study with the aim of providing protection to the liver and kidney, as increased levels of SGOT, SGPT, ALP, bilirubin, serum urea, BUN and creatinine were found in animals during *L. donovani* infection. Administration of the various doses of *A. racemosus* and *W. somnifera* significantly brought the levels of serum urea, BUN, creatinine, SGOT, SGPT, ALP and bilirubin within the normal range compared with infected controls, and no mortality was reported in infected animals treated with the herbal drugs. However, in the positive-control group of infected animals treated with SSG, there was an increase in the levels of SGOT, SGPT and creatinine. The probable mechanism that might be responsible for providing protection by *A. racemosus* is its antioxidant property and free-radical scavenging activity (Verma *et al.*, 2012). Kamat *et al.* (2000) also showed that a crude extract of *A. racemosus* has potent antioxidant properties against damage induced by gamma radiation in rat liver mitochondria (Ajith *et al.*, 2007). Moreover, an alcoholic extract of root of *A. racemosus* has been shown to significantly reduce the enhanced levels of SGOT and SGPT following carbon chloride-induced hepatic damage in rats (Kamat *et al.*, 2000). Jeyanthi & Subramanian (2009) investigated the nephroprotective effect of *W. somnifera* against gentamicin-induced nephrotoxicity in rats. They showed that *W. somnifera* at a dose of 500 mg kg$^{-1}$ significantly reversed the gentamicin-induced toxicity, which could be by enhancing antioxidant activity and scavenging the free radicals (Acharya *et al.*, 2012). Harikrishnan *et al.* (2008) reported that *W. somnifera* offered hepatoprotection by influencing the levels of lipid peroxidation products (due to the presence of alkaloids, withanoloids and flavonoids), its free-radical scavenging and antioxidant property (Jeyanthi & Subramanian, 2009).

In VL patients, the concentration of Hb has been found to be reduced along with an increased platelet count (Harikrishnan *et al.*, 2008). Major factors contributing to the anaemia during VL are haemylosis occurring in both the massively enlarged spleen and liver and haemodilution resulting from expansion of the plasma volume (Avasthi *et al.*, 2009). Thus, in the present study, two main haematological factors, Hb and TLC, were also investigated. In our studies, we also encountered anaemia along with a high TLC level in the infected animals. However, the levels of Hb and TLC were found to be in the normal range in all the infected animals treated with either SSG or the herbal drugs at different doses and combinations. This result was expected, as *W. somnifera* is used in the treatment of iron-deficiency anaemia due to its effects on haemtopoesis and natural iron content. It has also been found to enhance total WBC count, Hb and bone-marrow cellularity in children (Davis & Kuttan, 2000). Similarly, *A. racemosus* has also been found to significantly enhance total WBC count, haemoglobin content and bone-marrow cellularity in animals treated with cyclophosphamide, as it affects stem-cell differentiation (Begum & Anuradha, 2011).

In the current study, the standard drug, SSG, was used to compare the effectiveness of the two herbal drugs. Although the decrease in parasite load and increase in DTH were much better in the infected plus SSG-treated animals, SSG treatment produced abnormal changes in the levels of creatinine, SGOT and SGPT compared with infected mice treated with the herbal drugs, thus suggesting that *A. racemosus* and *W. somnifera* have promising anti-leishmanial activity with no side effects. Therefore, these herbal drugs are good alternative anti-leishmanial agents that could be used alone or together with the standard drugs to ameliorate their hepatotoxicity and to enhance the protective immune responses of patients.

**ACKNOWLEDGEMENTS**

The authors are grateful to the Indian Council of Medical Research for providing financial support under project no. 5/8-7(86)2010-ECD-II.

**REFERENCES**


