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IN VIVO EVALUATION OF THE ANTILEISHMANIAL ACTIVITY OF TWO IMMUNOMODULATORY PLANTS, *EMBLICA OFFICINALIS AND AZADIRACHTA INDICA* IN BALB/C MICE

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ABSTRACT
The available drugs for the treatment of leishmaniasis have been shown to have toxic effects which besides their efficacy reduction urge the development of new therapeutic agents capable of controlling this disease. In this sense, the immunomodulatory plants used in traditional medicine are gaining particular importance nowadays. The herbal plants *Emblica officinalis* as well as *Azadirachta indica* have strong immunomodulatory and medicinal properties. The current study was designed to evaluate the leishmanicidal effect of both the herbal plants alone or in combination. The control of infection was assessed in terms of Leishman Donovan Units (LDU) in the impression smears of liver. There was a significant reduction in parasite load in animals treated with herbal drugs in combination. DTH responses were measured as an index of cell-mediated immune response and were found to be higher in treated animals as compared to the infected controls, the most pronounced being in the animals treated with combination of herbal drugs. Higher levels of IgG2a and lower levels IgG1 levels were observed in this group of animals. The hematological and biochemical parameters were found to be normal in treated groups of animals.
Keywords: Visceral leishmaniasis, *Azadirachta indica, Emblica officinalis, Leishmania donovani*

INTRODUCTION

Leishmania parasites give rise to a number of different clinical manifestations. Visceral leishmaniasis (VL) is the most severe form and fatal if untreated. Aproximately 500,000 new VL cases are appearing every year, with an estimate of 50,000 deaths per year. Other types of the disease, cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and post-kala-azar dermal leishmaniasis (PKDL) are also major problems in many parts of the world[1]

Treatment is the most important tool for the control of disease. The available drugs against VL include sodium stibogluconate (Pentostam®) and meglumine antimoniate, amphotericin B deoxycholate, Fungizone® and AmBisome®. Miltefosine (Impavido®) has been approved as the first oral drug. Paromomycin is the latest drug to be registered for use in India against VL. The side effects associated with these drugs include significant toxicity, high cost and long treatment courses. The emergance of drug resistance has created the main hinderance for its control with one critical target in the state of Bihar in India[2]. Therefore, there is a need to search for cheaper, more effective, easily available and less toxic chemotherapeutic agents for combating leishmaniasis. The use of herbal preparations for the treatment of the disease holds a strong potential in that some ethnomedicinal plants have been demonstrated to contain potent leishmanocides. *Azadirachta indica* a member of Maliaceae family is used as an antimalarial[3], antibacterial[4] and antimicrobial[5]. It has also been shown to significantly improve the CD4+ cell count in HIV patients[6,7]. The crude methanol extracts of *A. indica* has also shown considerable in-vitro anti-leishmanial activity on *L. major* promastigotes with an LC50 of 10.2, 4.94, and 89.38 µg/ml respectively[8]. *In vitro* studies have also demonstrated its efficacy in eliminating *L. donovani* promastigotes after 48 h at concentrations of 0.1mg/ml[9].Another plant *Emblica officinalis* belonging to Euphorbiaceae family has been found to contain gallic acid, quercetin, flavonoids, glycosides, tannins and proanthocyanides and being used for the treatment of cancer,

diabetes, liver disorders, heart diseases, ulcers, diarrhea and dysentery etc. It also possesses anticancer, antioxidant, and immunomodulatory activities[10].

The present study was carried out with an aim to assess the leishmanicidal efficacy of two pure herbs, *E. officinalis* and *A. indica* in combination against murine visceral leishmaniasis and compare with conventional drug SSG (Sodium stibogluconate). It was assumed that these immunomodulatory herbs might enhance the immune system of the *L. donovani* infected BALB/c mice which may help in the killing of the parasite.

MATERIALS AND METHODS

PARASITE

The Indian strain of *Leishmania donovani*, viz. MHOM/IN/80/Dd8, originally obtained from the London School of Hygiene and Tropical Medicine, U. K. was used for the present study. The promastigotes of this strain were maintained in vitro at 22±1ºC in modified NNN medium by serial subcultures after every 48–72 hours[11].

ANIMALS

Inbred BALB/c mice (both sex), 5-6 weeks old weighing 20-25 gm were procured from Central Animal House of Panjab University, Chandigarh. They were fed with water and mouse feed *ad libitum*.

ETHICAL CLEARANCE

The ethical clearance for conducting the experiments was obtained from the Institutional Animal Ethics Committee, Panjab University, Chandigarh, India.

DRUGS

Azadirachta indica and *Emblica officinalis* as pure herbs were purchased from the Himalaya drug, Bangalore, India. These were dissolved in distilled water. Sodium stibogluconate (SSG) was purchased from Wellcome Research Laboratories, U.K. It was dissolved in distilled water in water bath at 72 ^{0}C to get the required concentration of 40 mg/kg b.wt. and was injected intraperitoneally into mice daily for 5 days[12].

INFECTION AND DRUG TREATMENT OF ANIMALS

BALB/c mice were infected intracardially with 1×10^7 promastigotes of *L. donovani.* After 30 post infection days, mice were treated with different doses of *Azadirachta indica* and *Emblica officinalis* for 15 days daily. The treated animals were divided into five groups.

Group A: Infected mice treated orally with *Emblica officinalis* alone at the dose of 100 mg/kg body wt. for 15 days daily.

Group B: Infected mice treated orally with *Azadirachta indica* alone at the dose of 100 mg/kg body wt. for 15 days daily.

Group C: Infected mice treated orally with combination of *Azadirachta indica* and *Emblica officinalis* at the dose of 100 mg/kg body wt. for 15 days daily.

Group D: Infected mice treated orally combination with *Azadirachta indica* and *Emblica officinalis* at the dose of 200 mg/kg body wt. for 15 days daily.

Group E: Infected mice treated intraperitoneally with SSG at the dose of 40 mg/kg daily for 5 days.

ASSESSMENT OF INFECTION

Six mice from different groups were sacrificed on 0, 7 and 15 post treatment days. Liver of each sacrificed animal was removed and weighed. Parasite load was assessed in the impression smears of liver in terms of Leishman Donovan Units by the method of Bradley and Kirkley[13]. Results were evaluated by comparing the parasite burden of treated animals with that of infected controls.

DELAYED TYPE HYPERSENSITIVITY (DTH) RESPONSES

All the groups of mice were challenged in the right foot pad with a subcutaneous injection of 40µl of leishmanin and left footpad with PBS, 2 days before the day of sacrifice. For preparing leishmanin, promastigotes in the stationary phase of growth were harvested from NNN medium and washed thrice with PBS (pH-7.2). The final pellet was suspended in 5ml of 0.5% (wt/v) phenol in sterile PBS and kept at room temperature for 10 minutes. Phenol was then removed and the final concentration was adjusted to $2x10^8$ promastigotes per ml. This was stored at 4ºC in aliquots. After 48 hours, the thickness of right and left foot pad was measured using a pair of vernier callipers. Results were expressed as mean ± S.D. of percentage increase in the thickness of the right foot-pad as compared to the left footpad of mice was calculated[14].

COLLECTION OF SERUM SAMPLES

Six mice from each group were anaesthetized on different post infection or post treatment days and blood was collected. The serum was separated for various biochemical tests and ELISA was carried out.

ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

The parasite specific IgG1 and IgG2a isotype antibodies were measured by ELISA by using commercially available kits (Bangalore Genei, Bangalore, India).

HEMATOLOGICAL INVESTIGATIONS

Hemoglobin was estimated in the blood by using Sahli's hemometer/ hemoglobinometer. Total leukocyte count (TLC) of all the mice was done on different post infection/post treatment days by the method of Khynriam and Prasad[15].

BIOCHEMICAL INVESTIGATIONS

Liver Function Tests. The estimation of Alkaline phosphatase (ALP), Serum Glutamate Oxaloacetate Transaminase (SGOT), and Serum Glutamate Pyruvate Transaminase (SGPT) was done in serum samples by using commercially available kits (Reckon Diagnostics Pvt. Ltd Baroda, India).

Kidney Function Tests. The estimation of bilirubin, blood urea nitrogen (BUN) and Creatinine was done in serum samples using commercially available kits (Reckon Diagnostics Pvt. Ltd Baroda, India).

STATISTICAL ANALYSIS

All the results were analyzed by student's t-test.

RESULTS AND OBSERVATIONS

HEPATIC PARASITE LOAD

In all the treated groups of animals parasite load decreased significantly ($p<0.001$) as compared to the infected controls. The reduction in parasite load was significantly ($p<0.001$) more in group A animals treated with *E. officinalis* as compared to group B animals treated with *A. indica*. The decrease in parasite load was more pronounced in group D as compared to group C animals. In both the combination groups the parasite load was ($p<0.0001$) lower in

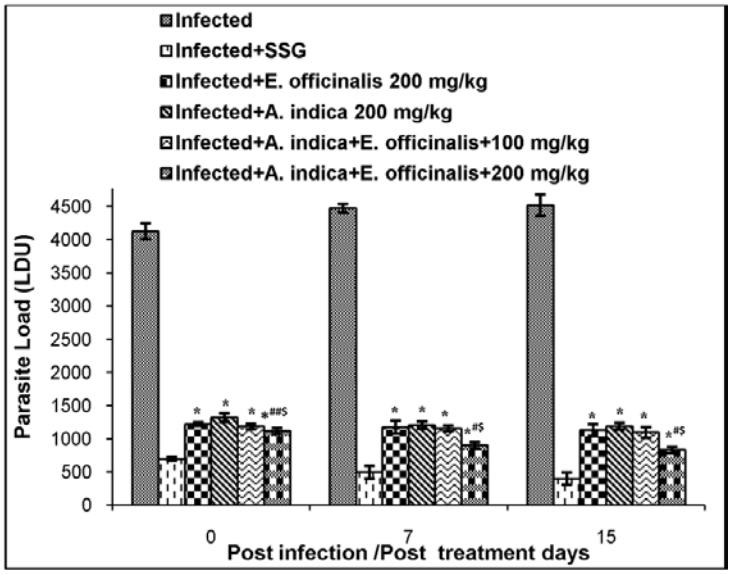

Fig 1 Parasite Load in various groups of animals. p value: Infected vs Infected+*E. officinalis*, 200 mg/kg, alone/Infected+*A. indica*, 200 mg/kg, alone / Infected+*E. officinalis*+*A. indica*, 100 mg/kg each/Infected+ *E. officinalis*+*A. indica*, 200 mg/kg, each. p value: Infected+*E. officinalis*+*A. indica*, 100 mg/kg, each vs Infected+ *E. officinalis*+*A. indica*, 200 mg/kg, each. p value: Infected+SSG vs Infected+ *E. officinalis*+*A. indica*, 200 mg/kg, each. $^{*}p<0.0001$, $^{\#}p<0.0001$, $^{\#\#}p<0.05$, $^{\$}p<0.0001$

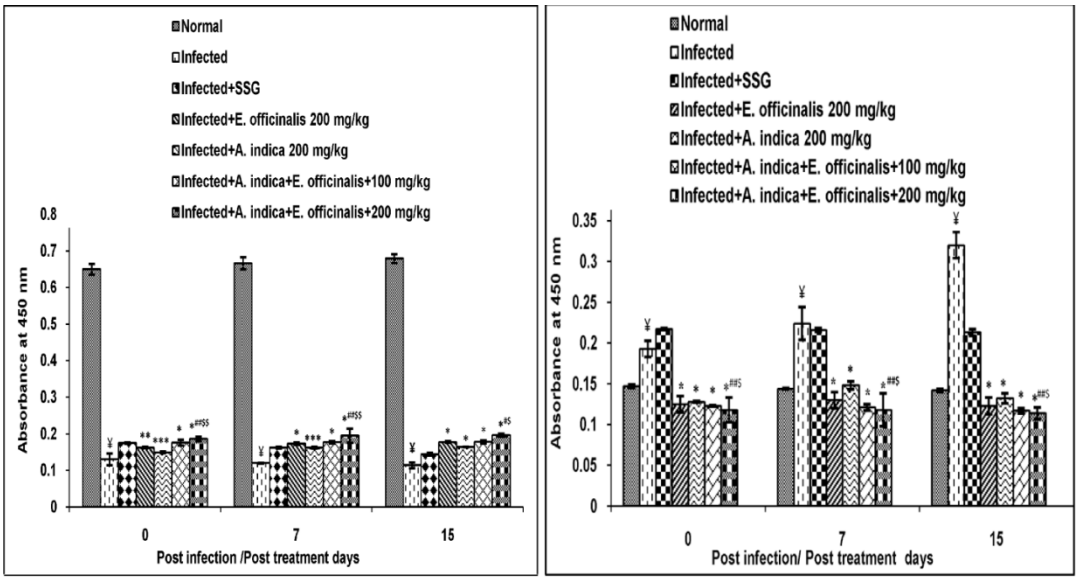

Fig 2 IgG2a and IgG1 antibody production in various groups of animals. p value: Normal vs Infected. p value: Infected vs Infected+*E. officinalis*, 200 mg/kg, alone/Infected+*A. indica*, 200 mg/kg, alone/ Infected+*E. officinalis*+*A. indica*, 100 mg/kg each / Infected+ *E. officinalis*+*A. indica*, 200 mg/kg, each. p value: Infected+*E. officinalis*+*A. indica*, 100 mg/kg, each vs Infected+ *E. officinalis*+*A. indica*, 200 mg/kg, each. p value: Infected+ SSG vs Infected+ *E. officinalis*+*A. indica*, 200 mg/kg, each. $^{¥}p<0.001$, $^{*}p<0.001$, $^{\#\#}p<0.05$, $^{\$}p<0.001$, $^{**}p<0.001$, $^{***}p<0.05$, $^{\#}p<0.001$,$^{\$\$}p<0.05$

significantly ($p<0.0001$) lower than the infected controls. In infected animals treated with SSG at a dosage of 40 mg/kg b.wt, (group E) the parasite load decreased significantly ($p<0.001$) as compared to group D animals (Fig 1).

HUMORAL IMMUNE RESPONSES

The levels of IgG1 antibody increased significantly ($p<0.001$) in infected animals as compared to normal controls on 30, 37 and 45 p.i.d. respectively. The levels of IgG1 antibodies decreased significantly ($p<0.0001$) in both A and B group animals as compared to the infected controls. The decrease was more pronounced in animals treated with *E. officinalis* as compared to *A. indica*. The absorbance values were significantly both combination significantly ($p<0.05$) lower in group D animals as compared to group C animals. However the absorbance values were significantly ($p<0.0001$) higher in group D animals as compared to group E animals (Fig 2).

The levels of IgG2a were significantly ($p<0.001$) higher in group A animals as compared to the infected controls on all post treatment days. Similarly the antibody levels were also significantly ($p<0.05$) higher in group B animals as compared to the infected controls. These antibodies were significantly increased in animals treated with *E. officinalis* as compared to *A. indica*. The levels of IgG2a antibody were significantly higher in both C and D groups of animals as compared to infected controls. The absorbance values were found to be higher in group D animals as compared to group C animals.

CELL MEDIATED IMMUNE RESPONSES

DELAYED TYPE HYPERSENSITIVITY (DTH) RESPONSES

The DTH responses increased significantly ($p<0.001$) in group A and group B animals as compared to the infected controls. The DTH responses were higher in animals treated with *E. officinalis* as compared to *A. indica*. The percentage increase in thickness was greater in group D than that observed in group C animals. In both the combination groups DTH responses were significantly ($p<0.0001$) increased than infected controls. In infected animals treated with SSG at a dosage of 40 mg/kg b.wt, the DTH responses increased significantly ($p<0.05$) as compared to the infected animals on all post treatment days (Fig 3).

HEMATOLOGICAL PARAMETERS

Hemoglobin (Hb) concentration decreased significantly ($p<0.01$) in infected animals as compared to normal animals on on all p.i.d. The Hb concentration was significantly higher ($p<0.01$) and was within normal range in all treated groups as compared to infected controls. The TLC also increased significantly ($p<0.001$) in infected animals as compared to normal animals on all p.i.d. It was found to be within normal range in all treated groups.

BIOCHEMICAL PARAMETERS

LIVER FUNCTION TESTS:

SGOT, SGPT and bilirubin activity increased significantly ($p<0.01$) and ALP activity decreased significantly ($p<0.01$) in infected animals treated with herbal drugs as compared to infected animals on all p.i.d. All the four liver function tests were found to be within normal range in all treated groups.

KIDNEY FUNCTION TESTS:

The concentration of creatinine, blood urea and blood urea nitrogen (BUN) increased significantly ($p<0.001$) in infected animals as compared to normal controls. In all the treated groups concentration of creatinine, blood Urea and BUN decreased significantly ($p<0.001$) as compared to the only infected group and were found to be within normal range.

DISCUSSION

Antimonials are still the main compounds used for treatment of VL but resistance, severe toxicity and parenteral administration are its limitations[16, 17]. The other drugs like Amphotericin B, AmBisome, miltefosine and Paromomycin are also not without serious side effects. Despite impressive cure rates for several antileishmanial agents, long hospitalization, resistance and toxicity of drugs has appealed to search for new therapies which include the use of herbal drugs[18].

Cure of the disease depends upon the development of an effective immune response that activates macrophages. During VL there is a profound immunosuppression in the host that promotes the survival of parasites. So combination therapy employing immunomodulators with antileishmanials can boost immune system to fight against this devious pathogen that evades the immune response by attenuating pro-inflammatory signaling pathways[19]. Therefore, essential prerequisites of an effective immunomodulatory, anti-leishmanial drug should be its potential to tilt the Th1-Th2 imbalance in favour of Th1[20]. Natural compounds that have shown promising antileishmanial activities are diverse in character and include phenolics, napthylisoquinoline alkaloids, sesquiterpene lactones including Luteolin, Quassin, *Aloe vera*, *Allium sativum*, *Tinospora sinensis*, Artemisinin and Berberine Chloride etc[21].

Azadirachta indica (Neem) has major concentration of these active compounds - azadirachtin, nimbin, nimbidin, nimbidol, sodium nimbinate and quercetin. It also contains many fatty acids like oleic acid, stearic acid, palmitic acid, linoleic acid and so on. It possesses immunostimulant, antimalarial, antioxidant, hepatoprotective,

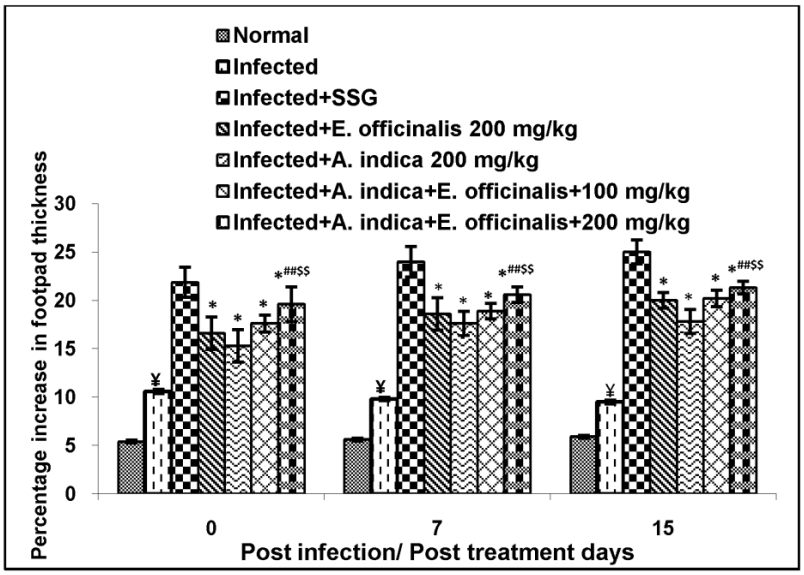

Fig 3 DTH responses in various groups of animals. p value: Normal vs Infected. p value: Infected vs Infected+*E. officinalis*, 200 mg/kg, alone/Infected+*A. indica*, 200 mg/kg, alone/Infected+*E. officinalis*+*A. indica*, 100 mg/kg each/Infected+*E. officinalis*+*A. indica*, 200 mg/kg, each. p value:Infected+*E. officinalis*+*A. indica*, 100 mg/kg, each vs Infected+ *E. officinalis*+*A. indica*, 200 mg/kg, each. p value: Infected+ SSG vs Infected+ *E. officinalis*+*A. indica*, 200 mg/kg, each. $^{¥}p<0.001$, $^{*}p<0.001$, $^{\#\#}p<0.05$, $^{\$\$}p<0.05$

anticarcinogenic, antipyretic and antiulcer activities[22]. Some studies have revealed that *A. indica* possesses antileishmanial activity. It eliminated *L. donovani* promastigotes in vitro after 48 h at concentrations of 0.1mg/ml[9].

Emblica officinalis (*amla*) has been known in Ayurvedic medicine for its tonifying, antiageing and immune enhancing properties[23] as it provides a superior source of vitamin C. it posseses a number of active compounds like ellagic acid, gallic acid, quercetin, flavonoids, glycosides and proanthocyanidins. Quercetin present in it is responsible for its hepatoprotective effect. *Amla* has been particularly indicated for anaemia, asthma, bleeding gums, diabetes, cold, chronic lung disease, hyperlipidaemia, yeast infections, scurvy and cancer[24, 25]. The present study was designed to study the antileishmanial effect of *A. indica* in combination with an immunomodulatory drug *E.*

Table 1 Hemoglobin concentration in various groups of animals

ANIMAL GROUPS	Hemoglobin concentration (in gms/dl)		
	0 p.t.d.	**7p.t.d.**	**15p.t.d.**
Normal	10.30±0.21	10.43±0.12	10.20±0.16
Infected	7.80±0.08	8.10±0.24	9.86±0.16
Infected+SSG	9.90±0.08	10.10±0.09	10.30±0.06
Infected+*E. officinalis* 200 mg/kg	10.60±0.16	10.70±0.08	10.36±0.26
Infected+*A. indica* 200 mg/kg	10.23±0.04	10.43±0.16	10.53±0.12
Infected+*A. indica*+*E. officinalis*+100 mg/kg	10.66±0.12	10.76±0.12	10.90±0.08
Infected+*A. indica*+*E. officinalis*+200 mg/kg	11.10±0.21	11.0±0.16	11.20±0.26

officinalis. It was proposed that immunomodulation by *E. officinalis* may boost up the antileishmanial activity of *A. indica*.

The parasite load declined significantly in all infected mice after treatment as compared to the only infected animals. However *A. indica* in combination with *E. officinalis* at the higher dose of 200 mg/kg b.wt, each, significantly reduced the parasite burden in infected animals and maximum reduction was seen in these animals as compared to lower dose of the pure herbs and also when the pure herbs were used alone. Our results are in correlation with the earlier study of antileishmanial efficacy of *A. indica* where it eliminated promastigotes *of L. donovani* after 48 hrs at concentrations of 0.1 mg/ml in vitro in BALB/c mice[9]. Among the two pure herbs, *E. officinalis* was more effective in reducing the parasite burden as compared to *A. indica*. Our study is also correlated with other studies in which Artemisinin reduced the parasite burden up to 86% in BALB/c mice infected with *L. donovani* (Sen *et al*., 2010). *Tinospora sinensis* inhibited the parasite load by 76.2±9.2% by enhancing ROS and NO production along with activation of macrophages[26].

In leishmaniasis, IgG2a and IgG1 kinetics indirectly reflect the Th1/Th2 responses. The relative production of these antibodies is used as a marker for the induction of protective (IgG2a induced Th1-type) or deleterious (IgG1 inducedTh2-type) type of immune responses. In the present study, all infected plus treated animals revealed higher levels of IgG2a and lower levels of IgG1 in comparison to the infected controls. The group D animals revealed maximum IgG2a with minimum IgG1 levels. The levels of IgG2a were greater in *E. officinalis* treated animals as compared to the animals treated with *A. indica.* The animals treated with SSG at the dose of 40 mg/kg b.wt, had lower levels of IgG2a and higher IgG1 in comparison to the animals treated with *A. indica* in combination with *E. officinalis* at a higher dose. It correlates with an earlier study which showed that SSG causes acute immunosuppression in the treated individuals[27]. In another study a neem leaf preparation induced a significant enhancement in the secretion of Th1 cytokine (IFN-γ) and a decreased secretion of Th2 cytokine (IL-10) against breast tumor antigen28. Since the levels of IgG1 and IgG2a points towards the generation of nonprotective Th2 and protective Th1 responses respectively, the results of the present study suggest that these pure herbs induced the generation of protective immune responses and the maximum protective efficacy was seen in the animals treated with the combination of these pure herbs in combination at a higher dose of 200mg/kg b.wt. each.

The DTH responses directly correlate with the cell-mediated immune (CMI) responses which potentiates the infiltration of lymphocytes and macrophages into the infected tissue for the clearance of pathogen from infected host[29]. Our results demonstrated that treated animals revealed increased DTH responses in comparison to the infected controls. It was greater in animals treated with *E. officinalis* as compared to those treated with *A. indica.* The DTH responses in animals treated with SSG at the dose of 40 mg/kg b.wt, were greater as compared to the animals treated with *A. indica* in combination with *E. officinalis* at the higher dose. Aqueous extract of dried *E. officinalis* resulted in increase in DTH responses in male Swiss Albino mice[30]. In the present study the infected animals treated orally with *A. indica* in combination with *E. officinalis* at the dosage of 200 mg/kg b.wt. revealed

Table 2 Total Leucocytes Count in various groups of animals

ANIMAL GROUPS	Total leucocyte count (per cubic mm)		
	0 p.t.d.	7 p.t.d.	15 p.t.d.
Normal	7348.66±83.92	7234.33±114.9	7229.66±99.06
Infected	10343.0±113.6	11666.0±111	14426.0±121.6
Infected+SSG	7500.0±8.95	7568.0±8.72	8000.0±7.95
Infected+*E. officinalis* 200 mg/kg	7673.3±110	7592.6±82.95	7513.3±102
Infected+*A. indica* 200 mg/kg	7726.66±89.93	7663.33±91.77	7650.0±173
Infected+*A. indica*+*E. officinalis*+100 mg/kg	7428.30±312	7176.6±281	7100.0±155.7
Infected+*A. indica* +*E. officinalis*+200 mg/kg	7283.30±217.6	7133.30±102.1	7073.30±131.2

maximum percentage increase in the thickness of footpad. The increased DTH responses in these animals points towards the efficacy of these pure herbs in the generation of cell-mediated immune responses.

One of the major presenting features in untreated visceral leishmaniasis is anemia[31], which is in accordance with our study where anemia was found in mice infected with *L. donovani*. In the present study, hemoglobin levels in all the treated groups increased and were in the normal range in comparison to infected controls. Infected animals treated orally with *A. indica* in combination with *E. officinalis* at the dosage of 200mg/kg b.wt. each, revealed maximum hemoglobin (Hb) levels and were in the normal range (Table 1)

Total leucocyte count in all the treated groups was reduced as compared to the infected controls and was within the normal range. However, maximum decline in TLC was observed when infected animals were treated orally with *A. indica* in combination with *E. officinalis* at the dosage of 200mg/kg b.wt. each and was within the normal range. Normal range of hemoglobin and TLC was 9.2-11.2 gm/dl and 7000-12000/mm^3 respectively. Infected animals treated with SSG at the dosage of 40 mg/kg b.wt. showed decrease in the levels of Hb and increased TLC in comparison to animals treated with these pure herbs in combination at a higher dose, but these were within the normal range (Table 2).

The present study correlates with previous studies which showed that SAG alone as well as SAG along with folic acid and vit. B12, improved the hemoglobin and TLC in all 50 cases of leishmaniasis[32]. The previous studies on the immunomodulatory potential of aqueous extract of *E. officinalis* in mice sensitized with SRBC's antigen showed normal hematological parameters at the doses of 100 and 200 mg/kg b.wt given orally for a period of 19 days[30]. A neem leaf preparation *in vitro* against tumor was observed to stimulate hematological systems as evidenced by the increase in total count of RBC, WBC, platelets and percentage hemoglobin[33]. Thus our study reveals that treatment of infected animals with both the herbs brought the levels of Hb and TLC in normal range as compared to the abnormal levels in infected animals.

Liver is the most important organ concerned with the biochemical activities in the human body. It has a great capacity to detoxicate toxic substances and synthesize useful principles. Therefore, damage to the liver inflicted by hepatotoxic agents is of grave consequence. Thus to assess the damage caused to the liver, various activities of enzymes like SGOT, SGPT, ALP and the concentration of bilirubin were measured. The increased level of SGPT, SGOT, ALP, and bilirubin is conventional indicator of liver injury. The stabilization of serum bilirubin, SGPT, SGOT, and ALP levels is a clear indication of the improvement in the functional status of the liver cells[34]. The increase in SGOT and SGPT levels has been reported in the VL patients[35]. In the current study there was no significant elevation in the levels of liver function tests in all the treated groups as compared to infected controls. All these were found to be in the normal range in all the treated groups of animals. But there was increase in the levels of SGOT, SGPT, ALP and serum bilirubin in infected animals treated with SSG at the dose of 40 mg/kg b.wt as compared to animals treated with *A. indica* in combination with *E. officinalis* at the higher dose Table 3,4,5 and 6). It indicates that SSG causes damage to the liver which correlates with the previous study which demonstrated that

Table 3 SGOT activity in different groups of animals

ANIMAL GROUPS	SGOT activity (U/L)		
	0p.t.d.	7p.t.d.	15p.t.d.
Normal	33.66±1.24	37.00±0.82	36.00±2.16
Infected	46.33±1.25	48.50±0.41	63.66±1.88
Infected+SSG	58.19±2.89	34.27±3.57	35.67±1.92
Infected+*E. officinalis* 200 mg/kg	30.90±3.68	30.50±0.82	30.80±0.96
Infected+*A. indica* 200 mg/kg	30.40±0.65	30.36±2.19	32.23±1.58
Infected+*A. indica*+*E. officinalis*+100 mg/kg	30.00±1.63	29.50±2.12	30.73±2.42

Infected+*A. indica*+*E. officinalis*+200 mg/kg	31.43±1.47	30.40±0.83	29.56±1.05

Table 4 SGPT activity in different groups of animals

ANIMAL GROUPS	SGPT activity (U/L)		
	0p.t.d.	7p.t.d.	15p.t.d.
Normal	32.36±0.74	33.23±0.85	34.10±0.73
Infected	44.80±1.20	46.93±1.10	52.76±1.75
Infected+SSG	57.98±1.92	43.99±1.67	40.67±2.50
Infected+*E. officinalis* 200 mg/kg	27.00±1.41	27.00±0.82	28.16±1.86
Infected+*A. indica* 200 mg/kg	25.40±3.58	29.06±0.89	27.66±1.25
Infected+*A. indica*+*E. officinalis*+100 mg/kg	26.35±3.94	22.83±4.40	22.20±1.65
Infected+*A. indica*+*E. officinalis*+200 mg/kg	27.76±1.92	24.16±1.64	23.66±3.85

the hepatic damage caused by SSG could be ameliorated by Picroliv which has hepatoprotective and immunostimulant activity[27]. Atangwho *et al*., showed that the extracts of *Vernonia amygdalina* and *Azadirachta indica* when administered in combination were seen to protect the liver as demonstrated by the normal activities of enzymes like SGOT, SGPT and ALP[36]. Achliya *et al*., reported that the oral administration of *Amalkadi Ghrita* (*Emblica officinalis, Glycyrrhiza glabra* and cow's ghee) was seen to lower significantly the levels of marker enzymes namely SGPT, SGOT, ALP and serum bilirubin which increased due to hepatic damage induced by CCl_4 in rats[34]. Both these studies correlate with our results and demonstrated a marked hepatoprotective effect of these pure herbs. Kidney function tests include estimation of urea, blood urea nitrogen and creatinine. Renal abnormalities caused by *Leishmania* have been well-documented in experimental animal studies and are comprised of interstitial and glomerular abnormalities[37]. There was no significant elevation in the levels of kidney function tests in animals treated with both the herbs as compared to the infected controls. These results were comparable with the animals treated with SSG. Infected animals treated with SSG revealed the increased concentration of creatinine suggesting the nephrotoxicity of the drug. Blood urea and BUN were also increased but they were in the normal range Table 7,

Table 5 Concentration of ALP in various groups of animals

ANIMAL GROUPS	ALP activity (KA)		
	0 p.t.d.	7p.t.d.	15p.t.d.
Normal	7.83±0.47	7.76±0.12	7.60±0.08
Infected	4.73±0.26	4.8±0.16	4.96±0.17
Infected+SSG	8.91±1.49	9.23±1.35	10.56±1.52
Infected+*E. officinalis* 200 mg/kg	7.76±0.21	8.00±0.21	8.60±0.49
Infected+*A. indica* 200 mg/kg	9.00±0.91	8.40±0.58	8.60±0.28

Infected+*A. indica*+*E. officinalis*+100 mg/kg	7.86±0.25	8.10±0.29	8.60±0.62
Infected+*A. indica*+*E. officinalis*+200 mg/kg	8.73±0.31	9.13±0.95	9.93±0.93

Table 6 Concentration of bilirubin in various groups of animals

ANIMAL GROUPS	Concentration of bilirubin activity (mg/dl)		
	0 p.t.d.	7p.t.d.	15p.t.d.
Normal	0.62±0.10	0.43±0.12	0.53±0.17
Infected	1.46±0.21	1.65±0.10	1.50±0.08
Infected+SSG	1.43±0.16	1.39±0.10	1.32±0.21
Infected+ *E. officinalis* 200 mg/kg	0.61±0.02	0.62±0.02	0.65±0.01
Infected+ *A. indica* 200 mg/kg	0.59±0.02	0.60±0.01	0.63±0.02
Infected+*A. indica*+*E. officinalis*+100 mg/kg	0.59±0.05	0.57±0.13	0.58±0.01
Infected+*A. indica*+*E. officinalis*+200 mg/kg	0.55±0.02	0.62±0.02	0.66±0.01

8 and 9). In the previous studies, Singh showed that cadmium and radiation produced toxic effect on kidney in mice and *Emblica* juice reduced these toxic effects[26]. Haque *et al*., revealed that the levels of serum urea and creatinine remained unaltered and normal architecture of the cortical and medullary parts of the kidney were also preserved after neem leaf preparation (NLP) treatment[33]. The present study demonstrated the nephroprotective potential of the pure herbs, since the levels of urea, blood urea nitrogen and creatinine were normal in these animals. Hence it proves the protective efficacy of these pure herbs.

Thus from the present study it is concluded that *A. indica* in combination with *E. officinalis* at a higher dose of 200 mg/kg b.wt, each, reduced the parasite load with an increase in DTH responses and protective antibody responses. Moreover these herbs do not cause any toxicity in the animals as suggested by normal biochemical parameters as compared to animals treated with SSG where increased levels of SGOT, SGPT, ALP etc. were found.

Table 7 Concentration of creatinine in different groups of animals

ANIMAL GROUPS	Creatinine concentration (mg/dl)		
	0p.t.d.	7p.t.d.	15p.t.d.
Normal	0.84±0.02	0.90±0.08	1.04±0.14
Infected	1.61±0.08	1.77±0.04	1.82±0.03
Infected+SSG	1.80±0.02	1.72±0.04	1.51±0.05
Infected+*E. officinalis* 200 mg/kg	0.74±0.04	0.84±0.04	0.78±0.02
Infected+*A. indica* 200 mg/kg	0.73±0.06	0.77±0.02	0.79±0.04
Infected+*A. indica*+*E. officinalis*+100 mg/kg	0.69±0.06	0.74±0.008	0.79±0.02

Infected+*A. indica*+*E. officinalis*+200 mg/kg	0.68±0.05	0.76±0.03	0.81±0.03

Table 8 Blood urea concentration in various groups of animals

ANIMAL GROUPS	Blood urea concentration (mg/dl)		
	0 p.t.d.	7p.t.d.	15p.t.d.
Normal	24.20±1.07	25.20±0.75	24.70±1.42
Infected	50.66± 2.71	55.33±3.96	62.93±4.68
Infected+SSG	43.80±0.79	39.80±0.65	32.87±0.25
Infected+*E. officinalis* 200 mg/kg	39.33±0.62	38.70±1.28	39.90±1.51
Infected+ *A. indica* 200 mg/kg	38.86±1.33	38.73±1.97	40.56±1.78
Infected+*A. indica*+*E. officinalis*+100 mg/kg	37.60±0.82	38.90±2.29	39.20±2.27
Infected+*A. indica*+*E. officinalis*+200 mg/kg	36.90±2.77	37.40±1.17	37.82±1.86

Table 9 BUN concentration in various groups of animals

ANIMAL GROUPS	BUN concentration (mg/dl)		
	0 p.t.d.	7p.t.d.	15p.t.d.
Normal	11.30±0.49	11.70±0.35	11.53± 0.66
Infected	23.65±1.27	25.84±1.85	29.38±2.18
Infected+SSG	20.80±0.82	18.75±0.76	16.55±0.59
Infected+*E. officinalis* 200 mg/kg	18.36±0.29	18.06±0.60	18.62±0.72
Infected+*A.indica* 200 mg/kg	18.14±0.62	18.08±0.92	18.93±0.83
Infected+*A. indica*+*E. officinalis*+100 mg/kg	16.90±0.45	17.10±1.07	18.08±1.06
Infected+*A.indica*+*E. officinalis*+200 mg/kg	16.23±1.29	17.80±0.55	18.06±0.86

Thus the potent antileishmanial and significant immunostimulatory activity of *A. indica* and *E. officinalis* indicates that these pure herbs may provide promising leads for the development of new drugs against leishmaniasis. Further investigations on other animal models, with various doses of these herbs in combination are required to completely eliminate the parasite from the host body without causing any side-effect.

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