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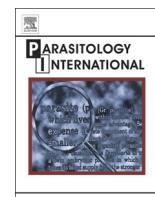


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Asparagus racemosus ameliorates cisplatin induced toxicities and augments its antileishmanial activity by immunomodulation *in vivo*

Heena Sachdeva ^a, Rakesh Sehgal ^b, Sukhbir Kaur ^{a,*}

^a Department of Zoology, Panjab University, Chandigarh 160014, India

^b Department of Parasitology, Post Graduate Institute of Medical Education and Research, Chandigarh 160014, India



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ABSTRACT

Current drugs for the treatment of visceral leishmaniasis are inadequate and their efficacies are also compromised due to suppression of immune function associated during the course of infection. To overcome this problem, efforts are needed to develop therapies with effective immunomodulatory agents where decrease of parasitic burden and simultaneous enhancement of adaptive immunity can be achieved. In this study we have evaluated a new therapeutic approach based on combination of *Asparagus racemosus*, an immunomodulatory drug, in combination with cisplatin against *Leishmania donovani* infected BALB/c mice. We demonstrate that *A. racemosus* (650 mg/kg b.wt./day for 15 days, orally) in combination with cisplatin (5 mg/kg b.wt./day for 5 days, intraperitoneally) enhanced the clearance of parasites as determined by Giemsa-stained liver impression smears. Besides having better killing activity, this combination group achieved increased production of disease resolving Th-1 response (IFN-gamma, IL-2), heightened DTH (delayed type hypersensitivity) response and augmented levels of IgG2a. Moreover, *A. racemosus* in combination with cisplatin not only provided enhanced protective immune response but also resulted in remarkable improved kidney and liver function tests as manifested by normal levels of SGOT, SGPT, alkaline phosphatase, creatinine and urea in blood plasma with normal histological observations as compared to only cisplatin treated *L. donovani* infected BALB/c mice. Through this study we have ascertained that *A. racemosus* in combination with cisplatin in *L. donovani* infected BALB/c mice boosted as well as restored both cellular and humoral immunity. Thus in view of severe immunosuppression in visceral leishmaniasis, a better and effective strategy for optimum efficacy of future antileishmanial drugs would direct not only killing of parasite by the drug, but also simultaneous generation of immunity against the disease.

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1. Introduction

Visceral leishmaniasis caused by *L. donovani* is a life-threatening disease involving uncontrolled parasitization of liver, spleen and bone marrow [1]. Control of visceral leishmaniasis (VL) relies exclusively on chemotherapy that includes pentavalent antimonials (SbV), pentamidine, amphotericin B deoxycholate (AB), lipid formulations of amphotericin B, paromomycin (PM) and orally effective, miltefosine (MF). All these drugs are limited to some extent by their toxicity, variable efficacy, inconvenient treatment schedules, requirement for hospitalization and/or cost. On the other hand, resistance against all drugs except amphotericin B, is prone to develop, as *Leishmania* has already developed resistance to two commonly used antileishmanials viz. SbV and pentamidine in India [2]. Although MF (hexadecylphosphocholine) is a promising orally active antileishmanial drug and currently used as first line regimen in the Indian subcontinent its long half life [3] and uncontrolled provision to patients may increase the likelihood of development of parasite resistance

[4]. Efficiency of *Leishmania* chemotherapy is also impaired due to suppression of immune function during the course of infection [5]. Disease outcome of VL is associated with various immunological dysfunctions. Both experimental [6] and clinical [7] data support a pathogenetic role of IL-10 in VL. Endogenous IL-10, known to suppress IFN-gamma synthesis, is capable of derailing Th1-type of immune response. Effective defense toward *L. donovani* infection depends strictly upon T (Th1) cells, and acquired resistance is governed by T cell and macrophage activating cytokines [8]. Therefore, an urgent need exists for the search for newer determinants which would in turn affect the disease's pathology through balanced beneficial immunomodulation of the host.

Cisplatin (*cis*-diamminedichloroplatinum II), currently one of the most important cytostatic agents in the treatment of a wide range of solid tumors [9], has been found to have antileishmanial activity. *In vitro* studies revealed its antileishmanial activity at a concentration of 0.25–64 µM. It was found that cisplatin induced a stage dependent cell cycle arrest at the S and G2 phase against promastigotes and axenic amastigotes [10]. *In vivo* antileishmanial activity of cisplatin has also been reported from our laboratory and it has been observed that treatment with low doses of cisplatin (0.5 mg and 1 mg/kg body wt.) in *L. donovani* infected BALB/c mice resulted in decreased parasite load

* Corresponding author at: Parasitology Laboratory, Department of Zoology, Panjab University, Chandigarh 160014, India. Tel.: +91 172 2541942; fax: +91 172 2541409.

E-mail address: puzoology@yahoo.com (S. Kaur).

and an initial transient and reversible increase in levels of SGOT, SGPT, BUN, blood urea, creatinine and phosphorus. Mild nephrotoxicity was also reported with 1 mg/kg body wt. of cisplatin. However, cisplatin did not cause complete elimination of the parasite at these doses [11]. Sharma et al. [12] evaluated the nephroprotective and immunomodulatory activity of various antioxidants in combination with cisplatin against murine visceral leishmaniasis. They found lesser parasite load in *L. donovani* infected BALB/c mice treated with cisplatin at the dose of 5 mg/kg b.wt. for five days daily, intraperitoneally as compared to those treated with 2.5 mg/kg b.wt. Also, combination of cisplatin with various antioxidants (Vitamins C and E and silibinin) resulted in increase of Th1 response (higher IgG2a, enhanced DTH response and greater concentration of IFN-gamma and IL-2) and normalization of various disturbed biochemical parameters as compared to only cisplatin treated animals.

Asparagus racemosus, commonly known as shatavari, has significant medicinal properties. It is used for the treatment of diarrhea, dysentery, rheumatism and nervous breakdown. The studies on the total extracts and the isolated principles have revealed a wide range of biological activities. These include antitumor [13], antifungal [14] and immunomodulatory [15] properties. The major active constituents are steroid saponins (Shatavarins) that are present in the roots. Other active compounds such as quercetin, rutin and hyperoside are found in flowers and fruits, while diosgenin and quercetin-3 glucuronide are present in the leaves. Maximum tolerable doses of 2000 mg/kg b.wt. with no toxicological consequences suggest that *A. racemosus* is safe to use [16]. It could enhance immunity through T-cells and has good humoral response. Steroidal saponins and steroid saponins (shatavaroside A and shatavaroside B) are major secondary metabolites present in *A. racemosus* that might be attributed to show immunomodulatory effects [17]. Oral administration of decoction of powdered root has been found to enhance the immunomodulatory effect [18].

Thus, keeping current status of visceral leishmaniasis treatment and the results obtained from our previous studies in mind, where cisplatin at low doses did not completely eliminated the parasite, use of *A. racemosus*, an immunomodulator, in combination with cisplatin has been approached in the present study for effective treatment of visceral leishmaniasis. This combination may aid in the antileishmanial efficacy of cisplatin by modulating immune responses of the host instead of using this drug alone at higher doses against visceral leishmaniasis which can lead to severe toxicities.

2. Materials and methods

2.1. Parasite

L. donovani promastigotes of strain MHOM/IN/80/Dd8, obtained from the London School of Tropical Hygiene and Medicine, London, were used for the present study and maintained in vitro at 22 ± 1 °C in modified Novy, McNeal and Nicolle's (NNN) medium by serial subcultures after every 48–72 h.

2.2. Animals

Experiments were performed on 4 to 6 week old inbred BALB/c mice of either sex weighing approximately 20 g. Mice were procured from the Institute of Microbial Technology, Chandigarh, India and prior approval for their use was obtained from the Institutional Animal Ethics Committee, Panjab University, Chandigarh, India. Mice were kept under standard conditions of temperature (25 ± 2 °C), 12 h light/dark cycle, fed with standard pellet diet and water *ad libitum*.

2.3. In vivo infection of mice

The promastigote culture in NNN medium was checked for bacterial or fungal contamination microscopically. The parasites in the logarithmic phase of growth were used for infection. The culture was pooled and

centrifuged at 2500 rpm for 15 min. The supernatant was discarded and the pellet (promastigotes) was washed 2–3 times in PBS and 1 ml final volume was made with the same. The promastigotes were counted in the Neubauer's chamber by diluting the promastigote suspension in 10% buffered formalin. These were then adjusted to a concentration of 10^8 parasites/ml. 0.1 ml of this suspension containing 10^7 parasites was injected intracardially into mice after mild ether anesthesia.

2.4. Experimental design

After 30 post infection days, mice were administered with cisplatin (Sigma-Aldrich, St. Louis, MO, USA) alone for five days daily and in combination with *A. racemosus* (Himalaya Drugs Company, Bangalore, India) for 15 days daily (five days along with cisplatin and then alone for ten days). In each group 21 mice were kept and 7 mice from each group were then sacrificed on 0, 15 and 30 post infection/post treatment days for various test procedures.

Group 1 n = 21	Normal control (PBS only)
Group 2 n = 21	Infected control (inbred BALB/c mice infected with 1×10^8 promastigotes of <i>L. donovani</i>)
Group 3 n = 21	Infected mice treated with cisplatin (at the dose of 5 mg/kg b.wt. for five days daily, i.p.)
Group 4 n = 21	Infected mice treated with cisplatin in combination with <i>A. racemosus</i> (at the dose of 650 mg/kg b.wt. for 15 days daily, p.o.)
Group 5 n = 21	Infected mice treated with <i>A. racemosus</i> alone (at the dose of 650 mg/kg b.wt. for 15 days daily, p.o.)

2.5. Assessment of infection

The course of infection (parasite load) was monitored by the microscopic examination of Giemsa-stained impression smears of liver. The parasite load was expressed as Leishman–Donovan units and was calculated as per the method of Bradley and Kirkley [19].

2.6. Cytokine assays by ELISA

It is well established that the cytokine milieu at the initiation of infection is critical in determining disease outcome. So to understand the interplay between the disease healing inflammatory cytokines IFN-gamma, IL-2 and disease associated cytokines IL-4 and IL-10, we sought to investigate the production of cytokines in infected and drug treated groups of BALB/c mice. The spleens from all the groups of mice were aseptically removed from all the groups of BALB/c mice and single cell suspensions were prepared in RPMI 1640 supplemented with 20 mM NaHCO₃, 10 mM HEPES, 10 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 10% FCS. RBCs were removed by lysis with NH₄Cl. The remaining cells were washed twice with culture medium and then cultured in triplicate in a 96 well flat bottom plate at a density of 2×10^5 cells/well in a final volume of 250 µl complete medium and stimulated with 50 µg/ml crude antigen (prepared from stationary phase promastigotes by giving 6 cycles of freeze and thaw to parasites/promastigotes). Promastigotes were then centrifuged at 10,000 rpm at 4 °C for 30 min. The supernatant containing antigen was collected and protein concentration was estimated.

The cells were then incubated for 72 h at 37 °C in a humidified chamber containing 5% CO₂. After 72 h incubation, culture supernatants were collected and stored at –20 °C for the assessment of concentration of IFN-γ, IL-2, IL-4 and IL-10 [20]. The supernatants were then analyzed for these cytokines by using cytokine ELISA kits according to the manufacturer's instructions (Diaclone, France and Bender MedSystems, Austria). The absorbance of each well was taken on an ELISA Reader (LISAPlus, India) at 450 nm.

2.7. Delayed type hypersensitivity (DTH) responses

Successful treatment protocol for the control of parasite multiplication is often related to antigen induced DTH response as an indication of activation of cell-mediated response. Therefore, DTH responses were determined as an index of cell mediated immune responses in all the groups of mice. The response was evaluated by measuring the difference in the footpad swelling. All groups of mice were challenged in the right foot pad with a subcutaneous injection of leishmanin and left foot pad with PBS [for preparing leishmanin, promastigotes in the stationary phase of growth were harvested from modified NNN medium and washed thrice with PBS (phosphate buffer saline)]. The final pellet was then suspended in 5 ml of 0.5% phenol in sterile PBS and kept at room temperature for 10 min. The phenol was then removed and the final concentration was adjusted to 2×10^8 promastigotes/ml]. After 48 h, the thickness of the right and left foot pads was measured by using a pair of vernier calipers. The percentage increase in the thickness of the right foot pad as compared to the left was calculated at 48 h following intradermal inoculation of the test footpad with subcutaneous injection of leishmanin and the swelling of the control (PBS injected) footpad with a pair of vernier calipers [21].

2.8. Assay of parasite-specific IgG1 and IgG2a isotypes by ELISA

Since the outcome of VL may be determined by the extent of immune system activation, it is especially important to characterize the changes in the immunoglobulins both during infection and drug treatment. IgG2a and IgG1 kinetics directly reflect the Th1/Th2 responses. The relative production of these isotypes can thus be used as a marker for the induction of Th1 or Th2 type of immune responses. We therefore analyzed production of these antibodies in all the groups of BALB/c mice. The serum specific immunoglobulin G (IgG) isotype antibody responses were measured by conventional enzyme-linked immunosorbent assay (ELISA). Dynatech 96-well ELISA plates were coated with crude antigen of *L. donovani* at a concentration of 200 ng/well. Sera from mice of all the groups were then added at two fold serial dilutions, followed by washes and addition of isotype specific HRP-conjugated secondary antibodies (rabbit anti-mouse IgG1 or IgG2a) after which the substrate and chromogen were added and absorbance was read on an ELISA plate reader at 450 nm [21].

2.9. Biochemical assays:

2.9.1. Liver function tests

SGOT (Serum Glutamate Oxaloacetate Transaminase), SGPT (Serum Glutamate Pyruvate Transaminase), bilirubin and alkaline phosphatase were measured to evaluate the liver function in serum samples of mice of all the groups by using commercially available kits (Reckon diagnostics Private limited, Vadodara, India).

2.9.2. Kidney function tests. As indicators of kidney function urea and creatinine were estimated in serum samples of mice of all the groups by using commercially available kits (Reckon diagnostics Private limited, Vadodara, India).

2.10. Histopathological analysis

Liver and kidney from all the groups of animals were removed and fixed in Bouin's fixative for 6 h and then the fixative was removed by keeping vials containing tissues covered with muslin cloth under the running tap water overnight. Then the tissues were dehydrated through ethanol series (70% to 100%), washed with xylene and then were embedded in paraffin wax. The thin sections of tissues were prepared with a microtome (5–7 μm) and then the sections were stained with hematoxylin-eosin stain. After that slides were examined and photographed using a light microscope.

2.11. Statistical analysis

All the experiments were performed three times independently. All data comparisons have been tested for significance by using two-way ANOVA followed by post hoc Tukey's test.

3. Results

3.1. Parasite load

Progressively *L. donovani* infected mice on treatment with cisplatin alone showed significant ($P < 0.0001$) reduction in the hepatic parasite load as compared to only infected BALB/c mice. However, treatment of *L. donovani* infected BALB/c mice with *A. racemosus* in combination with cisplatin induced most effective leishmanicidal activity as judged by the maximum elimination of liver parasites in this group as compared to only cisplatin treated infected BALB/c mice, however, on different p.t.d. no significant difference in the parasite load was observed between these groups of animals (Fig. 1).

3.2. Assay of cytokines in culture of splenocytes by ELISA

To understand the interplay between the disease healing inflammatory cytokines IFN-gamma, IL-2 and disease associated cytokines IL-4 and IL-10, we sought to investigate production of cytokines in different groups of BALB/c mice. It has been found that administration of *A. racemosus* in combination with cisplatin rendered a maximum significant increase in levels of IFN-gamma and IL-2 as compared to only cisplatin treated infected mice. However, minimum levels of IFN-gamma and IL-2 were found in only *L. donovani* infected BALB/c mice (Fig. 2A,B).

L. donovani infected mice showed maximum levels of IL-4 and IL-10. A significant decrease in the levels of IL-4 and IL-10 has been found in cisplatin administered *L. donovani* infected mice. However, maximum decrease was observed in *A. racemosus* in combination with cisplatin treated infected mice, however no significant difference was observed in the levels of IL-4 between the groups infected mice treated with cisplatin and infected mice treated with cisplatin in combination with *A. racemosus*. Since, IL-4 and IL-10 are Th-2 specific cytokines, the minimum levels of these cytokines in the above said group suggest the suppression of Th2 response (Fig. 2C,D).

3.3. Delayed type hypersensitivity response

Chemotherapeutic intervention and cure are generally associated with the acquisition of a delayed-type hypersensitivity (DTH) response and consequently "classical" cell-mediated immunity. Therefore, the effect of administration of *A. racemosus* in combination with cisplatin in infected mice on antigen specific cellular immune response was measured by determining the degree of DTH response using the foot paw swelling test. Results of the current study revealed that infected BALB/c mice treated with cisplatin showed significantly ($P < 0.0001$) higher DTH responses compared to only infected mice. However maximum DTH response was observed in *A. racemosus* in combination with cisplatin treated infected mice (Fig. 3).

3.4. Assay of IgG2a and IgG1 levels by ELISA

Since the outcome of VL may be determined by the extent of immune system activation, it is especially important to characterize the changes in the immunoglobulin ratios both during infection and drug treatment. It is well known that the cytokines such as IFN- γ and IL-4 direct immunoglobulin class switching of IgG2a and IgG1, respectively. We therefore analyzed production of these antibodies in all the groups of mice. It was observed that sera from infected mice treated with *A. racemosus* in combination with cisplatin regimen showed significantly enhanced IgG2a ($P < 0.001$), a surrogate marker for Th1, in comparison to

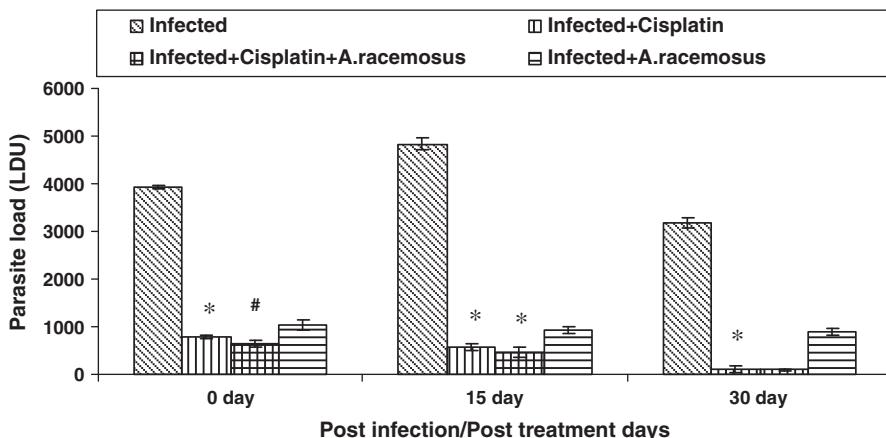


Fig. 1. Parasite load in various groups of BALB/c mice. P value: Infected control vs infected + cisplatin treated vs infected + *A. racemosus* + cisplatin treated vs infected + *A. racemosus* treated. *P<0.0001, #P<0.01, P>0.05 (non-significant).

only cisplatin treated infected mice, however no significant difference was observed on 0 p.t.d. This humoral response was also maintained in only *A. racemosus* treated infected BALB/c mice. Moreover, IgG1, a surrogate marker for Th2 cell differentiation, was elevated particularly in *L. donovani* infected BALB/c mice (Fig. 4A,B).

3.5. Liver function tests

The protective effect of *A. racemosus* on cisplatin induced hepatotoxicity was monitored by estimating the levels of SGOT, SGPT, ALP and bilirubin. It has been found that administration of cisplatin in *L. donovani* infected BALB/c mice showed a significant ($P<0.0001$) increase in the activity of SGOT and SGPT on different post treatment days respectively as compared to infected controls. However, results of the present study indicate that administration of *A. racemosus* in combination with cisplatin rendered a protection against cisplatin toxicity as was evident from the lowered levels of both SGOT and SGPT. Their activity was found to be in the normal range in other groups of BALB/c mice. Activity of other markers of the liver such as serum alkaline phosphatase and serum bilirubin was found to be in normal range in all the groups of animals (Table 1).

3.6. Kidney function tests

Parallel to hepatotoxicity, cisplatin administration in *L. donovani* infected BALB/c mice resulted in deranged kidney function as characterized by increase in concentration of serum urea and creatinine as compared to infected controls. However, treatment with *A. racemosus* in combination with cisplatin markedly reversed levels of serum urea and creatinine. In other groups of animals these levels were found to be in the normal range (Table 2).

3.7. Histopathological studies

The morphological features of hematoxylin and eosin stained sections of the kidney of *L. donovani* infected BALB/c mice were similarly near to control with intact glomerulus, proximal and distal convoluted tubules but with some clusters of lymphocytes. The section of the kidney of cisplatin treated mice showed lymphocytic infiltration and loss of tubular distinction. However, the kidney sections of infected mice treated with *A. racemosus* in combination with cisplatin revealed normal basic kidney structure (Fig. 5).

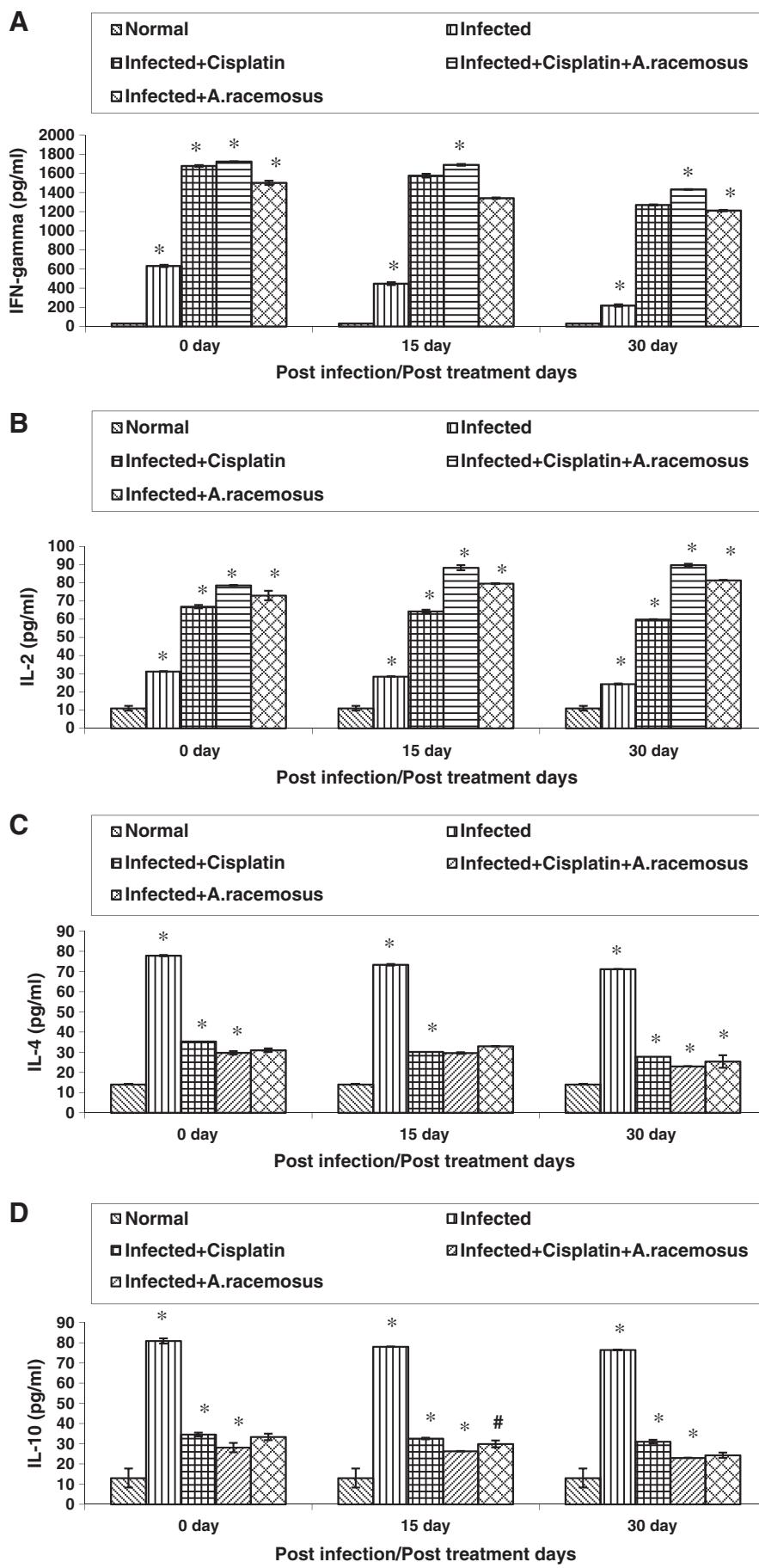
Liver sections of *L. donovani* infected BALB/c mice showed Kupffer cell hyperplasia. In the liver sections of infected mice treated with cisplatin microvesicular fatty change and vacuolization were observed. However *A. racemosus* in combination with cisplatin ameliorated liver injury and retained normal liver morphology (Fig. 6).

4. Discussion

It is well documented that the immune system synergistically aids to the therapeutic efficacy of antiparasitic drugs [22]. The use of immunomodulators in combination with conventional chemotherapy to enhance host immune responses has several advantages as a means to improving current therapeutic regimens. Therefore, an antileishmanial drug that can effectively and quickly reverse the immunosuppression of the infected host, besides killing the parasite, is desirable. In the present study we have explored the adjunct effect of *A. racemosus* on the efficacy of cisplatin using *L. donovani* infected BALB/c mice. The major findings emerging from this study are that the combination of *A. racemosus* with cisplatin results in maximum clearance of *L. donovani* from the liver of established and chronically infected BALB/c mice as compared to only cisplatin treated *L. donovani* infected BALB/c mice. However, only marginal level of parasites persists in liver. Belkaid et al. [23] also showed that complete clearance of parasites did not occur from liver when BALB/c mice infected with high dose of *L. donovani* parasites were treated with anti-IL-10 Ab in combination with pentavalent antimonials or AmB. Reduction in parasite load was also found in *L. donovani* infected BALB/c mice treated with *A. racemosus* alone. Earlier studies suggested that the possible mode of maximum parasite killing by the involvement of *A. racemosus* may be due to a potent antileishmanial molecule, racemoside A, present in the fruits of *A. racemosus*. This compound showed an IC₅₀ value of 1.31 µg/ml against promastigotes of *L. donovani* and exerted its antileishmanial effect through the induction of programmed cell death mediated by loss of plasma membrane integrity and loss of mitochondrial membrane potential culminating cell cycle arrest [24].

Visceral leishmaniasis is characterized by a variety of immunopathological consequences in man. The most remarkable of these are depression of cell mediated response and B cell activation [25]. Bhattacharjee et al. [26] stated that Th1 response from cytokines IFN-gamma and IL-2 is protective for VL whereas expression of Th2 cytokines viz. IL-4 and IL-10 increases during infection. Supportive evidence for the confirmation of the above mentioned facts was obtained from our studies as

Fig. 2. Cytokine levels in culture supernatants of spleen cells in various groups of BALB/c mice, A) interferon-gamma; B) interleukin-2. C) Interleukin-4 D) Interleukin 10. P value: normal control vs infected control vs infected + cisplatin treated vs infected + *A. racemosus* + cisplatin treated vs infected + *A. racemosus* treated. *P<0.0001, #P<0.01, P>0.05 (NS—non significant).



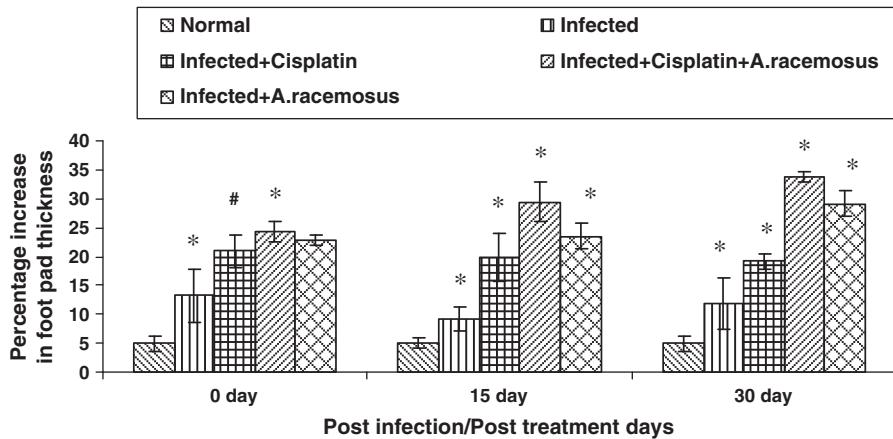


Fig. 3. Percentage increase in foot pad thickness (delayed type hypersensitivity) in various groups of BALB/c mice. P value: normal control vs infected control vs infected + cisplatin treated vs infected + *A. racemosus* + cisplatin treated vs infected + *A. racemosus* treated. *P<0.0001, #P<0.01, P>0.05 (non-significant).

combination of *A. racemosus* and cisplatin treatment generated a protective immunity through induction of IFN-gamma, IL-2 and decline in IL-4 and IL-10, which suggestively are responsible for preventing relapse and reinfection.

Delayed type hypersensitivity reaction is a type IV hypersensitivity reaction that develops when antigen activates sensitized T_{DTH} cells. Activation of T_{DTH} cells by antigen through appropriate antigen reaction results in secretion of IFN-gamma and IL-2 [27]. Secreted cytokines recruit macrophages and promote enhanced phagocytic activity for

more effective killing of parasites. Thus DTH reaction is important in host defense system against parasite. The importance of a positive DTH response in human leishmaniasis is illustrated by the fact that apparent clinical cure in the absence of a positive DTH response is often predictive of a relapsing infection [28]. Our results demonstrated that the DTH response was depressed in *L. donovani* infected BALB/c mice as compared to only cisplatin treated *L. donovani* infected BALB/c mice. However, treatment with *A. racemosus* in combination with cisplatin generated maximum DTH response as compared to only

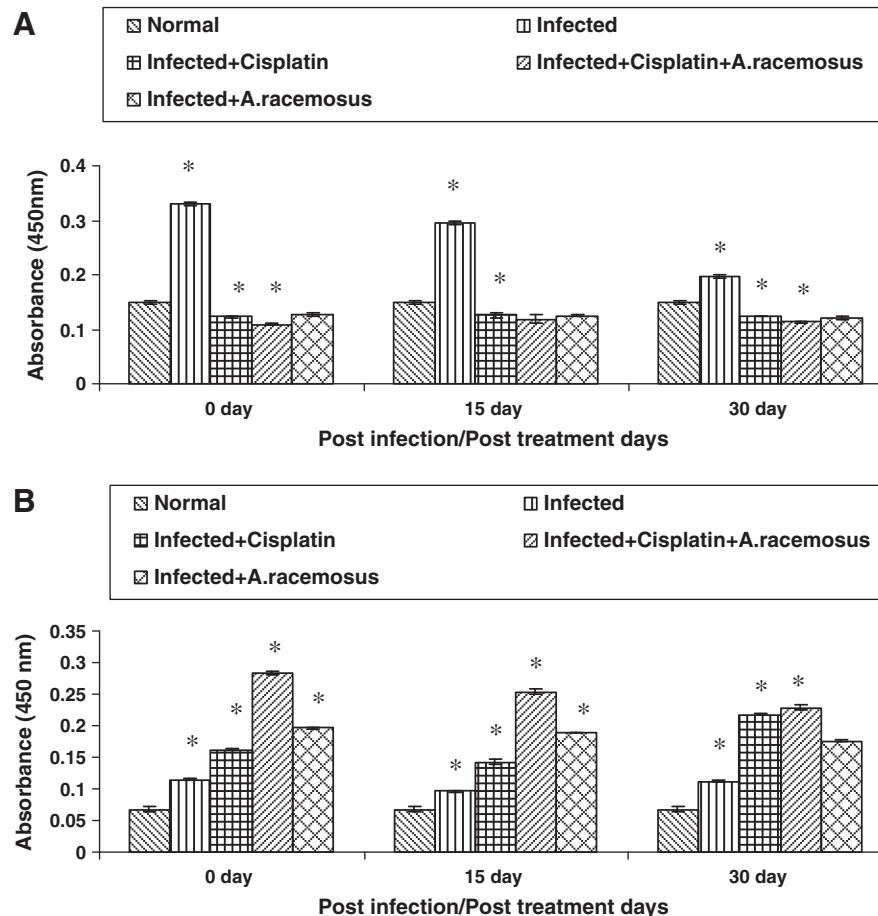


Fig. 4. Levels of antileishmanial isotype specific antibodies in serum samples of various groups of BALB/c mice, A) immunoglobulin G1; B) immunoglobulin G2a. P value: normal control vs infected control vs infected + cisplatin treated vs infected + *A. racemosus* + cisplatin treated vs infected + *A. racemosus* treated. *P<0.0001, #P<0.01, P>0.05 (non-significant).

Table 1
Estimation of liver function tests.

Groups	SGOT ^a			SGPT ^b			Bilirubin ^c			ALP ^d		
	0 day	15th day	30th day	0 day	15th day	30th day	0 day	15th day	30th day	0 day	15th day	30th day
Normal	37.8 ± 0.3	37.8 ± 0.3	37.8 ± 0.3	34.2 ± 0.2	34.2 ± 0.2	34.2 ± 0.2	0.37 ± 0.03	0.37 ± 0.03	0.37 ± 0.03	7.77 ± 0.1	7.77 ± 0.1	7.77 ± 0.1
Infected	98.3 ± 1.2*	67.7 ± 1.7*	43.9 ± 1.4#	43.9 ± 0.5*	43.9 ± 1.3*	41.3 ± 0.7*	0.27 ± 0.01*	0.31 ± 0.03#	0.19 ± 0.01*	5.5 ± 0.2*	4.1 ± 0.1*	4.1 ± 0.1*
Infected + cisplatin	168.8 ± 3.9*	143.05 ± 0.9*	119.2 ± 2.8*	232.2 ± 0.1*	189.3 ± 0.7*	144.2 ± 3.2*	0.14 ± 0.01*	0.15 ± 0.01*	0.13 ± 0.04#	6.2 ± 0.4*	4.2 ± 0.4#	4.2 ± 0.4#
Infected + cisplatin + A. racemosus	33.6 ± 0.1*	38.2 ± 3.4*	37.1 ± 0.1*	34.3 ± 0.7*	35.0 ± 0.3*	28.7 ± 0.1*	0.17 ± 0.02*	0.19 ± 0.01#	0.15 ± 0.03#NS	6.3 ± 0.1*	6.9 ± 0.01*	6.9 ± 0.01*
Infected + A. racemosus	39.8 ± 2.1*	39.0 ± 5.6#NS	37.8 ± 1.3 NS	29.1 ± 0.2*	37.4 ± 0.3*	34.9 ± 2.8*	0.18 ± 0.04#NS	0.21 ± 0.03*	0.32 ± 0.01*	5.0 ± 0.02*	5.3 ± 0.2*	5.4 ± 0.3#

*P < 0.0001, #P < 0.01, P > 0.05, NS—non-significant.
^a Estimation of SGOT (Serum Glutamate Oxaloacetate Transaminase) in various groups of BALB/c mice. P value: normal control vs infected control vs infected treated vs infected treated with A. racemosus in combination with cisplatin vs infected treated with A. racemosus alone.
^b Estimation of SGPT (Serum Glutamate Oxaloacetate Transaminase) in various groups of BALB/c mice. P value: normal control vs infected control vs infected treated with A. racemosus alone.

^c Estimation of bilirubin in various groups of BALB/c mice. P value: normal control vs infected control vs infected treated with A. racemosus alone.

^d Estimation of ALP (Alkaline Phosphatase) in various groups of BALB/c mice. P value: normal control vs infected control vs infected treated with A. racemosus alone.

cisplatin and only A. racemosus treated L. donovani infected BALB/c mice respectively.

Further to assess the immunological status of the animals we also evaluated the parasite specific IgG1 and IgG2a levels and it was found that L. donovani infection in BALB/c mice resulted in increased IgG1 and decreased IgG2a levels. However, L. donovani infected BALB/c mice treated with A. racemosus in combination with cisplatin showed maximum decrease in IgG1 with maximum increase in IgG2a levels as compared to only cisplatin and only A. racemosus treated L. donovani infected BALB/c mice respectively. The increased levels of IgG2a in the combination group serve as an indicator of Th1 response and hence act as a marker for natural resistance to visceral leishmaniasis. The study also supports the view that though leishmanicidal activity of cisplatin is direct, A. racemosus in combination with cisplatin has the capability to induce immunomodulation, thus imparting additional effects toward antileishmanial activity of cisplatin. Our results comply with the reports of Gautam et al. [29] who showed that oral administration of aqueous extract of A. racemosus at 100 mg/kg per day dose for 15 days resulted in a significant increase in antibody titers in experimental animals immunized with diphtheria, tetanus and pertussis vaccine. Gautam et al. [30] also showed the upregulation of Th1 and Th2 cytokines after administration of A. racemosus aqueous root extract (ARE) in SRBC sensitized animals, thus, suggesting Th1/Th2 adjuvant activity. ARE treated animals also resulted in significant increase in DTH response, antibody titres and CD3+, CD4+/CD8+ percentages indicating toward its effect on T cell activation.

The impairment of kidney function by cisplatin is recognized as the main side effect and the most important dose limiting factor associated with its clinical use. There is a continuous search for agents which provide nephroprotection against the renal impairment induced by drug like cisplatin for which allopathy offers no remedial measures. It is thus imperative that we turn toward alternative system of medicine. As renal damage starts within an hour after administration of cisplatin, it is important that a protective agent is present in sufficient concentration in renal tubules as injury occurs [31]. So, in the current study A. racemosus was administered along with cisplatin in L. donovani infected BALB/c mice as noticeable elevations in creatinine and urea levels in plasma of only cisplatin administered infected mice were observed. The histopathological changes in the kidney also supported the biochemical findings as damaged brush border epithelium of distal and proximal convoluted tubules and lymphocyte infiltration were seen in cisplatin treated infected mice. However A. racemosus in combination with cisplatin normalized raised creatinine and urea levels. Also, histopathological changes in kidney preparations of A. racemosus and cisplatin treated infected mice allow normalization of kidney architecture not greatly different from that in normal mice.

Cisplatin administration in infected mice also resulted in prominent hepatotoxicity as confirmed by biochemistry and histopathology. Deterioration of liver function viz. increased levels of SGOT, SGPT and histopathological changes like vacuolization and microvesicular fatty change in liver morphology confirmed hepatic dysfunction. However, A. racemosus in combination with cisplatin has been shown to prevent liver alterations as normal levels of SGOT, SGPT and normal hepatic morphology were observed in this group. Hence, A. racemosus in combination with cisplatin in infected mice was found to afford nephroprotection as well as hepatoprotection. The probable mechanism that might be responsible for providing protection against cisplatin induced toxicity by A. racemosus is its antioxidant property and free radical scavenging activity because formation of free radicals and depletion of antioxidant enzymes are some of the pathogenic mechanisms of cisplatin induced toxicity [32,33]. Supportive evidence for confirmation of the our above mentioned results was obtained by Kamat et al. [16] who showed that crude extract of A. racemosus has potent antioxidant properties against damage induced by gamma radiation in rat liver mitochondria. Moreover, alcoholic extract of root of A. racemosus has been

Table 2
Estimation of kidney function tests.

Groups	Creatinine ^a			Urea ^b		
	0 day	15th day	30th day	0 day	15th day	30th day
Normal	1.3 ± 0.1	1.3 ± 0.2	1.3 ± 0.2	29.6 ± 0.1	29.6 ± 0.3	29.6 ± 0.3
Infected	1.3 ± 0.3 ^{NS}	1.4 ± 0.1 ^{NS}	1.3 ± 0.1 ^{NS}	23.3 ± 0.3*	77.1 ± 0.01*	38.96 ± 0.1*
Infected + cisplatin	10.0 ± 0.7*	9.3 ± 0.3*	7.0 ± 0.1	129.7 ± 0.4*	94.11 ± 0.1*	76.0 ± 0.02*
Infected + cisplatin + <i>A. racemosus</i>	1.39 ± 0.4*	1.2 ± 0.01*	1.3 ± 0.3	41.3 ± 0.01*	43.0 ± 0.2*	44.9 ± 0.1*
Infected + <i>A. racemosus</i>	1.4 ± 0.3 ^{NS}	1.3 ± 0.02*	1.2 ± 0.01	44.6 ± 0.1*	45.9 ± 0.3*	43.1 ± 0.4*

*P < 0.0001, P > 0.05, NS—non-significant.

^a Estimation of serum creatinine in various groups of BALB/c mice. P value: normal control vs infected control vs infected and cisplatin treated vs infected treated with *A. racemosus* in combination with cisplatin vs infected treated with *A. racemosus* alone.

^b Estimation of serum urea in various groups of BALB/c mice. P value: normal control vs infected control vs infected and cisplatin treated vs infected treated with *A. racemosus* in combination with cisplatin vs infected treated with *A. racemosus* alone.

shown to significantly reduce the enhanced levels of SGOT and SGPT in carbon chloride induced hepatic damage in rats [34].

5. Conclusion

Taken together, these findings indicate that treatment of infected mice with cisplatin in combination with *A. racemosus* significantly prevented the cisplatin caused nephrotoxicity and hepatotoxicity with further reduction in parasite load and increased production of Th1 cytokines (IFN-gamma and IL-2). Cure as well as acquired resistance against *L. donovani* infection was due to the direct parasite-

killing activity of cisplatin and switching-on of Th1-based protective cell-mediated immunity by *A. racemosus*. Thus, cisplatin in combination with *A. racemosus* can emerge as a prospective antileishmanial therapy owing to its safety and ability to enhance disease healing Th1 immune responses.

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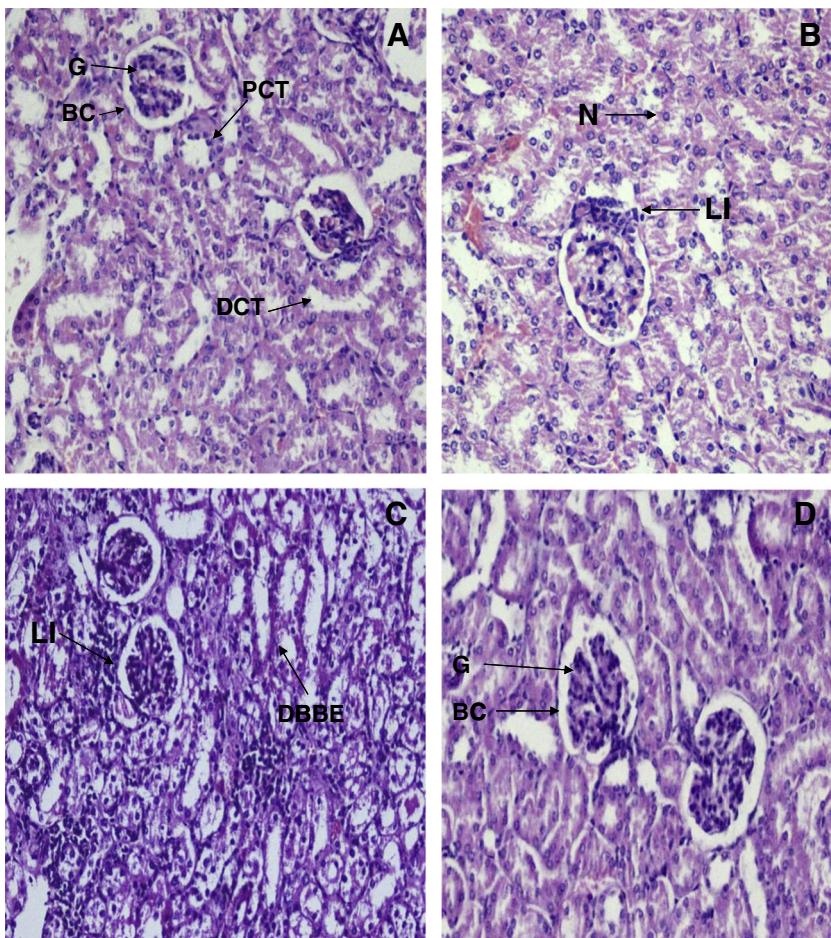


Fig. 5. Transverse section of kidney of BALB/c mice stained with hematoxylin and eosin stains (400×). A) Normal control. B) Infected control. C) Infected mice treated with cisplatin alone. D) Infected mice treated with *A. racemosus* along with cisplatin (Abbreviations: BC—Bowman's capsule, G—glomerulus, PCT—proximal convoluted tubule, DCT—distal convoluted tubule, LI—lymphocytic infiltration, DBBE—damaged brush border epithelium, N—nucleus).

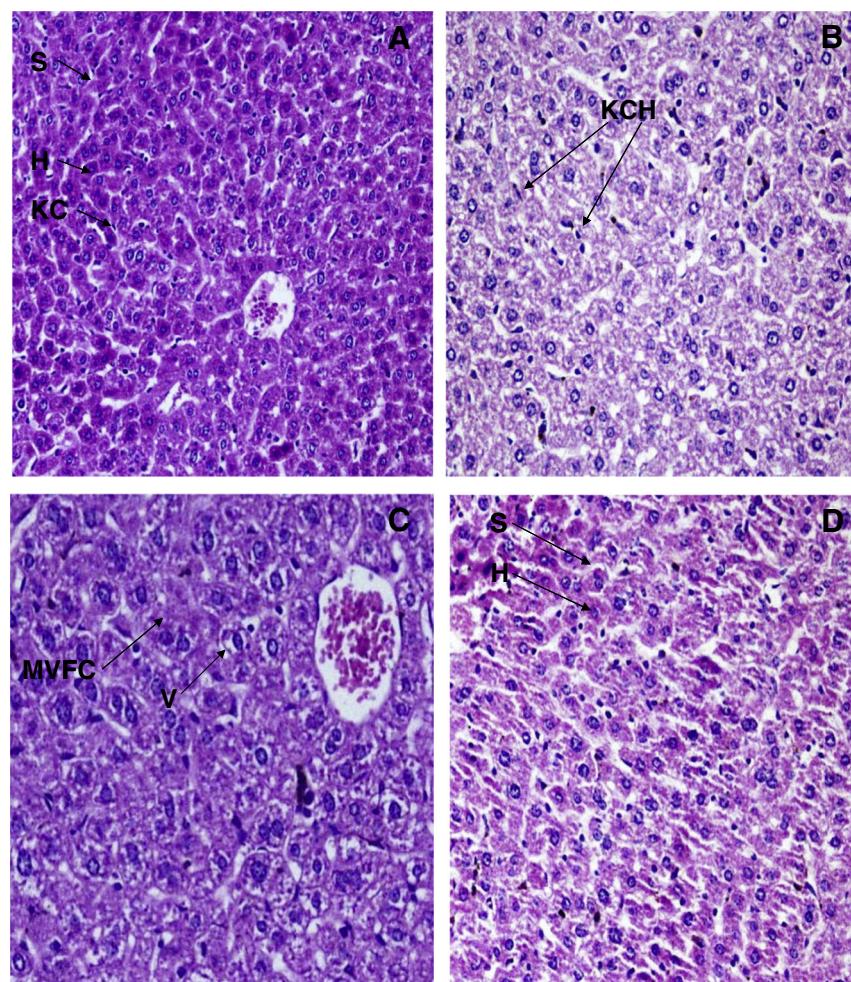


Fig. 6. Transverse section of liver of BALB/c mice stained with hematoxylin and eosin stains ($400\times$). A) Normal control. B) Infected control. C) Infected mice treated with cisplatin alone. D) Infected mice treated with cisplatin along with *A. racemosus* (Abbreviations: H—hepatocytes, KC—Kupffer cells, S—sinusoids, MVFC—microvesicular fatty change, V—vacuolization, KCH—Kupffer cell hyperplasia).

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