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Original Article

EVALUATION OF THE ANTILEISHMANIAL EFFICACY OF MEDICINAL PLANT *CHENOPODIUM ALBUM* LINN. AGAINST EXPERIMENTAL VISCERAL LEISHMANIASIS

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ABSTRACT

Objective: Visceral leishmaniasis is a neglected tropical disease resulting in death if not properly treated. The ongoing search for better leishmanicidal compounds has brought herbal drugs into the limelight as safe and effective substitutes to conventional therapies which have various drawbacks. The current study was designed to evaluate the antileishmanial efficacy of medicinal plant *Chenopodium album* Linn. against *Leishmania donovani* in inbred BALB/c mice.

Methods: Inbred BALB/c mice were infected intravenously with $1x10^7$ *Leishmania donovani* promastigotes and kept for 30 d. These animals were then treated with two doses (500 mg/kg body wt. and 1000 mg/kg body wt.) of methanolic extracts of *C. album* orally for 7 d.

Results: The animals treated with methanolic extracts of *C. album*, revealed a significant reduction in parasite load. These animals also showed heightened delayed type hypersensitivity (DTH) response and increased IgG2a as an indicator of protective Th1 type of immune response. Moreover, the liver and kidney function tests were found to be in the normal range.

Conclusion: Hence the drug proved to be a good antileishmanial, but further studies recommended before it is to be tested in higher animal models.

Keywords: Visceral leishmaniasis, *Chenopodium*, BALB/c, DTH

INTRODUCTION

Visceral Leishmaniasis (VL; Kala-azar) is a vector-borne disease caused by protozoan parasite *Leishmania donovani*. It is a life-threatening infectious disease affecting around 500,000 and killing 50,000 individuals a year [1]. 90% of the cases are concentrated in the parts of India, Brazil, Sudan, Nepal, Ethiopia and Bangladesh. The disease is characterized by fever, pallor, hepatosplenomegaly, lymphadenopathy, immunosuppression and progressive weakness in the patient. It may lead to death if untreated [2]. A large number of drugs are available to treat the disease, but unfortunately, available drugs for the treatment have been shown to have toxic effects, which besides their efficacy reduction due to the emergence of parasite-resistant strains [3], urges the development of new therapeutic agents capable of controlling this disease [4,5]. Moreover till date, there is no commercial vaccine against any human parasitic disease including leishmaniasis. The absence of an antileishmanial vaccine and an ideal and cheap drug to reverse the immunosuppression poses a major challenge for VL elimination in the Indian subcontinent and worldwide [6].

Therefore, in the sparse inventory of antileishmanial drugs and the emergence of resistance, it is high time to implement strategies which could overcome drug unresponsiveness. An immuno-modulatory drug with anti-leishmanial functions which not only kills the parasite but also promotes the host protective immune response is necessary for the treatment of this dreaded disease [4, 5]. A search is on for new drugs that are less toxic, easily available and within the reach of poor people most afflicted by the disease [7]. In this sense, traditional medicine is gaining particular importance nowadays [4, 5]. Numerous plant derived products from different structural classes have been investigated as antileishmanial candidates including various alkaloids, terpenoids, flavonoids and quinonoid [8].

The genus *Chenopodium* Linn. (family-Chenopodiaceae) is a native plant of Western Asia. In India, the plant is well known for its 21 species some of which are also used as a source of food and grains. *C album* Linn. is commonly used for food and medicinal values; viz bathu sag, parupkkirai etc and grows in waste places and as a weed in wheat or other crops [9]. The plant is widely neglected herb with various pharmacological properties like antiviral, antimalarial, immunomodulatory, anti-allergic etc [10, 11]. It has been found to have flavonoids as phenolic amide, saponin, cinnamic acid amide, xyloside, phenols and lignin, etc. [12-15]. Essential oils extracted from *C. ambrosioides* (alpha-terpinene, p-cymene, and ascaridole) exhibited similar antileishmanial activities against intracellular amastigote form, with IC50 values between 4.7 and 12.4 µg/ml [16]. The methanolic extract of *C album* has been shown to have anticancerous activity and positive results have been obtained with the successful control of the growth of cells [17]. Alcoholic and aqueous extracts of *C album* have been reported to restore the integrity of hepatocytes significantly and reversed the integrity of alcohol induced side effects [18]. These findings demonstrated that *C album* has immense potential as a medicinal plant. Thus, with an urge to find a safe, natural and cheap antileishmanial drug that is not toxic and can cause a reversal of immunosuppression, the present study has been designed to evaluate the antileishmanial activity of *Chenopodium album* in inbred BALB/c mice against visceral leishmaniasis.

MATERIALS AND METHODS

Parasite

The strain of parasite used for the present study was MHOM/IN/80/Dd8 originally obtained from London School of Tropical Hygiene and Medicine, U. K. The promastigotes of these strains were maintained in RPMI-1640 medium with 10% fetal calf serum (FCS).

Plant material

Chenopodium album as the whole plant was collected from roadside near Dhanas lake, Chandigarh, India. The identification was confirmed from the Department of Botany, Panjab University, Chandigarh, India and number were taken (voucher number: 1445). Whole plants of *C. album* were washed thoroughly with water and dried at room temperature and then powdered.

Preparation of the extracts

In the present study, leaves and fruits of *Chenopodium album* were used for extraction. They were washed thoroughly with distilled water, dried at room temperature for few days and then ground

using a blender. The methanolic extract of the leaves and fruits was prepared by Soxhlet extraction method. The crude extract was obtained after filtration with Whatman's filter paper and evaporated under low pressure in a rotary evaporator. The sample was then lyophilized and the condensed extract stored at 4 °C for further studies

Preparation of the standard drug

Sodium stibogluconate (SSG) was purchased from Sigma-Aldrich Co., USA and required concentration of 40 mg/kg b. wt was prepared by dissolving it in distilled water at 72ºC in water bath [19].

***In vivo* antileishmanial activity of plant extract**

Animals

Inbred BALB/c mice (5-6 w old) were purchased from the Institute of Microbial Technology and maintained at Central Animal House of Panjab University, Chandigarh. Animals were allowed to acclimatize to the laboratory conditions for at least 2 w before experimental manipulation. All mice were maintained on a 12-hour light/dark cycle with free access to water and food *ad libitum* and were housed 6 mice per cage. The permission for conducting experiments was obtained from Institutional Animal Ethics Committee of Panjab University, Chandigarh (Approval no-IAEC/284-295).

Infection and treatment of animals

The animals were infected with 1 x 10^7 promastigotes of *L. donovani* intravenously. After 30 d post infection, mice were treated with two different doses (500 mg/kg body wt and 1000 mg/kg body wt.) of *C album* for 7 d orally. These drugs were prepared by dissolving in standard suspension vehicle (SSV). SSV was prepared by mixing carboxymethyl cellulose (5g), benzyl alcohol (5 ml) and tween-80 (4 ml) in 0.9% aqueous sodium chloride (1000 ml). Mice receiving Phosphate-buffered saline (PBS) only served as normal controls. Infected untreated mice were considered as infected controls, and the treated controls consisted of uninfected mice receiving either of the two doses (500 mg or 1000 mg/kg body weight) of the plant extract. SSG was given intraperitoneally at a dose of 40 mg/kg body wt. for 5 d continuously.

Groups of animals

All animals were divided into seven groups. Each group contained 12 mice and six mice in each group were sacrificed on 1 and 15 d post treatment.

Group I: Normal mice

Group II: Infected mice

Group III: Infected mice treated with SSG

Group IV: Normal mice treated with *Chenopodium album* (500 mg/kg body wt.)

Group V: Infected mice treated with *Chenopodium album* (500 mg/kg body wt.)

Group VI: Normal mice treated with *Chenopodium album* (1000 mg/kg body wt.)

Group VII: Infected mice treated with *Chenopodium album* (1000 mg/kg body wt.)

Assessment of infection:

Six mice from each group were sacrificed on 1 and 15 d. p. t. (days post treatment). Impression smears of liver were made on clean, grease free slides, fixed in methanol and stained with giemsa. The parasite load was calculated in the form of Leishman-Donovan Units by the method of Bradley and Kirkely [20], and calculated as follows:

$$LDU = \frac{\text{No. of amastigotes}}{\text{No. of macrophages}} X \text{ Weight of organ (mg)}$$

Delayed-type hypersensitivity (DTH) responses

The mice were given a subcutaneous injection of leishmanin in the right footpad and PBS (control) in the left footpad. The leishmanin was prepared by according to the method of Joshi *et al.*, [21].

Humoral immune response

The levels of IgG1 and IgG2a antibodies were measured by indirect ELISA in serum samples of different groups of animals [19].

Evaluation of biochemical parameters

Liver function tests

Serum Glutamate Oxaloacetate Transaminase (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT), Alkaline liver phosphatase (ALP) and bilirubin were tested by using commercially available kits (Reckon Diagnostics Pvt. Ltd. Vadodra, India) [21].

Kidney function tests

Urea and creatinine were estimated by using commercially available kits (Reckon Diagnostics Pvt. Ltd. Vadodra, India).

Statistical analysis

The student's t-test was performed to calculate the p-value for significance; p-values<0.05 were considered significant. Results were expressed as mean±standard deviation (SD) of three independent experiments.

RESULTS

Hepatic parasite load

The parasite load was measured in terms of LDU. In the case of the infected animals treated with *C. album*, the parasite load was significantly (p<0.05) lowered as compared to the infected animals.

However, the reduction in parasite load was more in animals treated with 1000 mg/kg body wt. of *C. album* (1220.05±55.70 and 1160. 89±47.61) as compared to 500 mg/kg body wt (1101.90±40.27 and 990.78±33.45) of *C. album* on 1 and 15 d. p. t. respectively (fig. 1). The LDU in the infected animals treated with SSG at a dose of 40 mg/kg body wt. was found to be 687.21±27.21and 501.76±15.98 on 1 and 15 d. p. t. respectively.

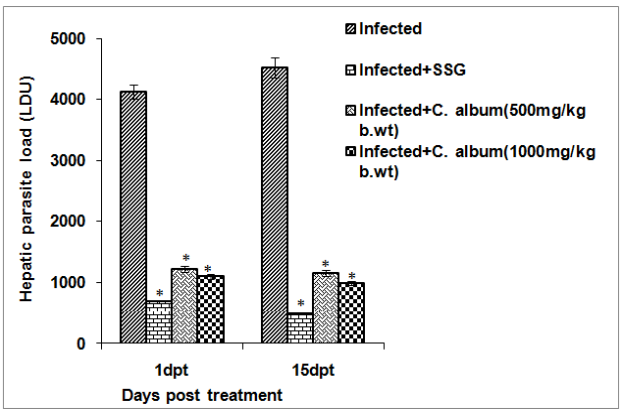

Fig. 1: Parasite load in various groups of BALB/c mice

*p-value: Infected vs all the groups p<0.05, Results were expressed as mean±SD of three independent experiments

Delayed-type hypersensitivity responses

In the infected animals treated with *C album* at a dose of 500 mg/kg body wt, DTH was found to be 36.67±1.89 and 41.87±1.85 on 1 and 15 d. p. t. respectively. These responses augmented with an increase in the dose of the drug and post treatment days (p<0.05) i.e. 38.45±1.26 and 42.54±1.76 on 1 and 15 d. p. t in infected and treated animals with 1000 mg/kg body wt. of *C album* (fig. 2). The DTH responses were greater in infected animals treated with SSG as compared to the animals treated with *C album*.

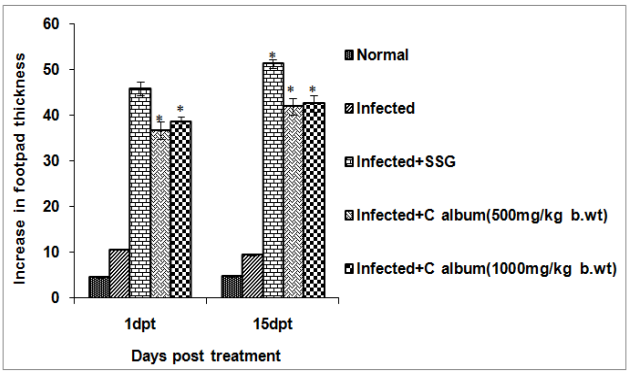

Fig. 2: DTH responses in various groups of BALB/c mice

*p-value: Infected vs all the groups p<0.05, Results were expressed as mean±SD of three independent experiments

Humoral immune responses

Antibody responses in different groups of animals were analyzed for isotype specific IgG1 and IgG2a antibodies in the sera of animals. Treated animals reveal greater IgG2a (p<0.05) and lesser IgG1 levels in comparison to the infected controls (p<0.05). Maximum IgG2a levels were observed in the animals treated with *C album* at a higher dose pointing towards the generation of the protective Th1 immune responses (fig. 3 and fig. 4).

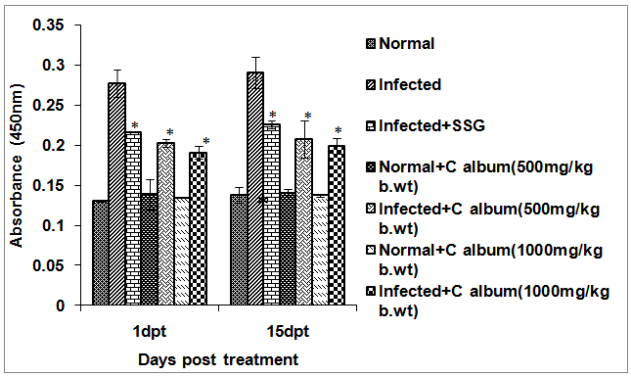

Fig. 3: IgG1 antibody levels in various groups of BALB/c mice

*p-value: Infected vs all the groups p<0.05, Results were expressed as mean±SD of three independent experiments

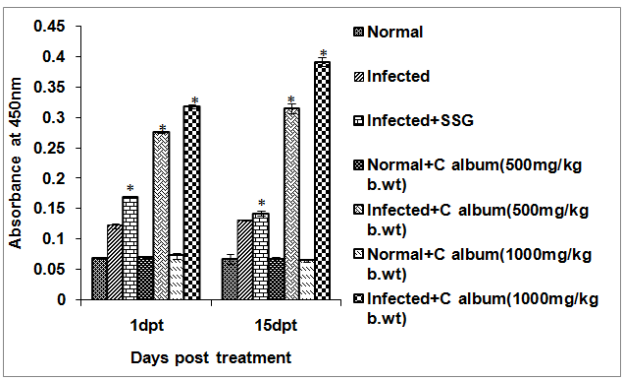

Fig. 4: IgG2a antibody levels in various groups of BALB/c mice

*p-value: Infected vs all the groups p<0.05, Results were expressed as mean±SD of three independent experiments

Biochemical parameters

Liver function tests

The levels of SGOT, SGPT, ALP and bilirubin were estimated in the serum samples of all the groups of mice. The concentration of all these enzymes were higher in the infected animals as compared to all the other groups (p<0.05) in which normal levels of these liver function tests were observed. The infected animals treated with SSG revealed the elevated levels of SGOT and SGPT in the serum samples (fig. 5, fig. 6, fig. 7 and fig. 8).

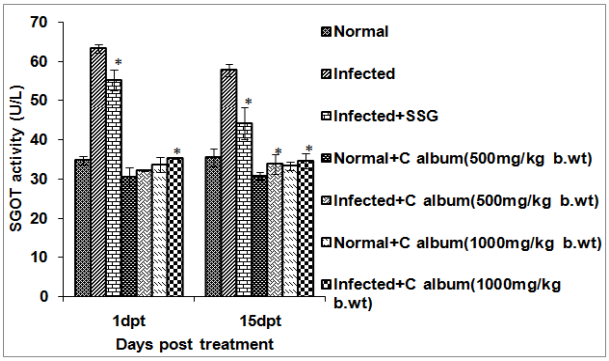

Fig. 5: SGOT activity in various groups of BALB/c mice on different days post treatment

*p-value: Infected vs all the groups p<0.05, Results were expressed as mean±SD of three independent experiments

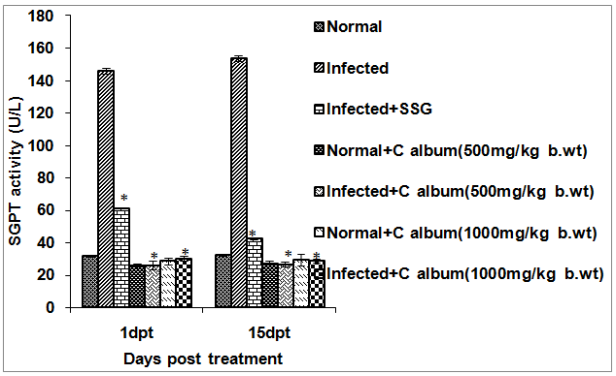

Fig. 6: SGPT activity in various groups of BALB/c mice on different days post treatment

*p-value: Infected vs all the groups p<0.05, Results were expressed as mean±SD of three independent experiments.

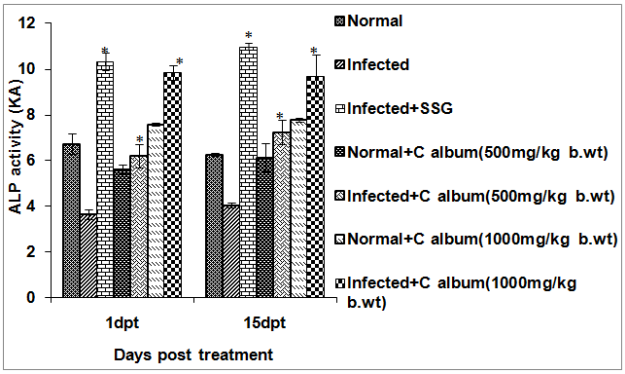

Fig. 7: Alkaline Liver Phosphatase (ALP) activity in various groups of BALB/c mice on different days post treatment

*p-value: Infected vs all the groups p<0.05, Results were expressed as mean±SD of three independent experiments

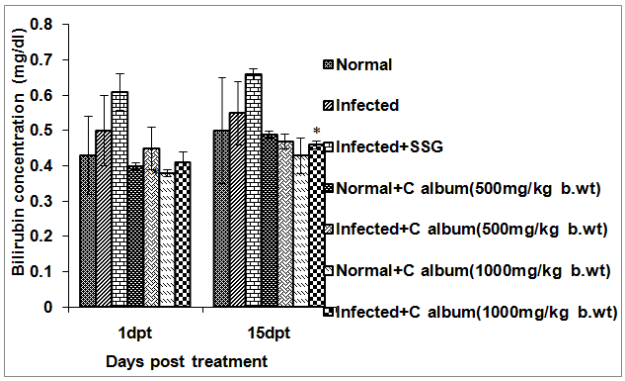

Fig. 8: Bilirubin concentration in various groups of BALB/c mice on different days post treatment

*p-value: Infected vs all the groups p<0.05, Results were expressed as mean±SD of three independent experiments

Kidney function tests

Kidney function tests include estimation of urea and creatinine. The concentration of serum creatinine and blood urea was observed to be in the normal range in all the groups except in the infected controls.

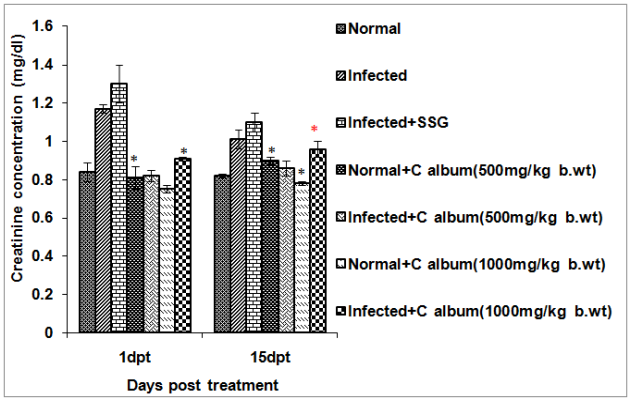

Fig. 9: Creatinine concentration in various groups of BALB/c mice on different days post treatment

*p-value: Infected vs all the groups p<0.05, Results were expressed as mean±SD of three independent experiments

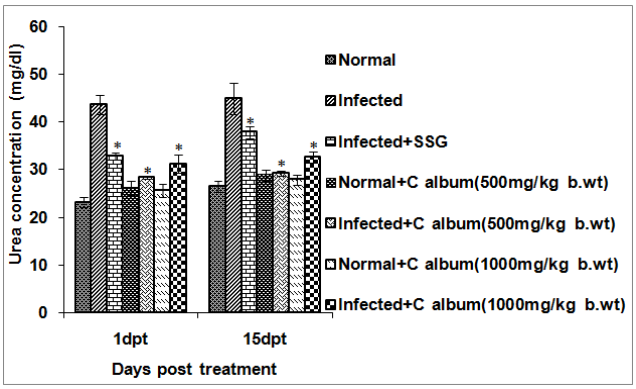

Fig. 10: Urea concentration in various groups of BALB/c mice on different days post treatment

*p-value: Infected vs all the groups p<0.05, Results were expressed as mean±SD of three independent experiments

Though the levels of creatinine in the animals treated with SSG were in the normal range, they were higher as compared to the normal animals and to the animals treated with the herbal extracts, indicating that SSG is nephrotoxic at higher concentration. Blood urea and creatinine levels were found to be in the normal range in the animals treated with *C album* on all days post treatment (fig. 9 and fig. 10).

DISCUSSION

Despite the wealth of information regarding the genetics of the parasite and experimental immunology of the disease, there is currently no licensed vaccine against *Leishmania* and control measures rely on chemotherapy to alleviate the disease and on vector control to reduce the transmission [22]. Most of the drugs employed for treatment are toxic, marginally effective, given by injection or compromised by the development of resistance. Since safe, effective and affordable chemotherapeutic agents for leishmaniasis are clearly needed, the identification of new antileishmanial candidates is an urgent priority.

Therefore in view of the present clinical scenario, it is desirable that new drugs should be developed with lesser cost, lower toxicity, and more effectiveness. Thus, the present study was carried out to evaluate the leishmanicidal properties of *Chenopodium album* in inbred BALB/c mice against visceral leishmaniasis.

Resistance to *Leishmania* infection depends on intracellular parasite killing by activated macrophages through the production of activated nitric oxide (NO) pathways. In our present study, the parasite count was observed minimum in treated animals as compared to the infected animals. However, maximum reduction was seen in animals given a higher dose of crude extracts (1000 mg/kg body wt.) as compared to the lower dose of the crude extract of *C. album*. Our results are in synchrony with the studies conducted by Makwali *et al.*, [23] using a single dose of saponin, plumbagin, trifluralin and acriflavine resulted in 97.8%, 98.8%, 86.4% and 85.4% reductions in liver amastigotes burden on day 7 post-treatment respectively. In a similar study, *Tinospora sinensis* reduced the parasite load by 76.2±9.2% by enhancing reactive oxygen species (ROS) and NO production along with the activation of macrophages [24]. In our study, although the reduction in parasite load in animals treated with *C. album* was lesser than those treated with SSG, the other medicinal properties of this plant and also the fact that it could be administered orally gives an added advantage, and thus this drug can be considered as an alternative drug.

A complex balance between the parasite and the genetic/ immunological background of the host are decisive for infection evolution and clinical outcome. Delayed-type hypersensitivity is an immunologic response that has frequently been used as a correlation for protection against or sensitization to *Leishmania* antigens in human and experimental models of *Leishmania* infection [25]. Chemotherapeutic cure of leishmaniasis is largely dependent upon the development of an effective immune response that activates macrophages to produce toxic nitrogen and oxygen intermediates to kill the amastigotes. In the present study, DTH response to leishmanin after treatment with *C. album* was evaluated by the percentage increase in thickness of food. Our results demonstrated that treated animals with a higher dose revealed increased DTH responses in comparison to the infected controls pointing towards the efficacy of this drug in generating an effective T cell-mediated immune response.

In the mouse, it has been shown that antibodies of the IgG1 isotype are produced mainly during a Th2 immune response, and IgG2a antibodies are produced mainly during Th1 immune responses [26]. In the present study, ELISA showed higher levels of protective IgG2a antibodies and lower levels of disease progressive IgG1 antibodies in the infected animals treated with an extract of *C album*. IgG2a is a Th2 supported subclass characterized by an early IFN-γ production and inducible NO synthase by activated macrophages. In contrast, IgG1 supports the protective Th1 type of immune response characterized by an early burst of interleukin-4 expression and succumbs to progressive visceral infection [27]. Similar results were observed in our laboratory, where *L donovani* infected animals

treated with the extracts of *Azardirachta indica* and *Emblica officinalis* showed higher levels of IgG2a and lower IgG1 levels in comparison to the infected controls [28].

Kidney function is an indication of the state of kidney and its role in renal physiology. The results of kidney tests are important in assessing the excretory function of kidneys. In the present study, we estimated the creatinine and urea concentration in the serum samples of infected as well as treated animals. In all the animals treated with *C album,* normal levels of urea and creatinine were observed. This suggests that drug does not cause any toxicity. Similarly, liver function tests are helpful screening tool and an effective modality to detect hepatic dysfunction. They were performed by assessing the levels of liver marker enzymes (SGOT, SGPT and ALP) and the concentration of bilirubin in the serum samples of mice. The present study revealed the elevated levels of hepatic enzymes indicative of liver damage on the administration of SSG. However, these levels were found to be in the normal range in animals treated with extracts of *C album*. Thus, the results demonstrated a marked hepatoprotective effect of the herbal plant. Our results are in corroboration with a study by, Nigam and Prakash [18] in which they observed a hepatoprotective potential of *C album* against alcohol-induced liver damage and found that the crude aqueous and alcoholic extracts showed restoration of serum transaminases, alkaline phosphatases, and bilirubin content and did not show any sign of toxicity up to an oral dose of 5g/Kg in mice.

CONCLUSION

The primary criteria of searching for new drugs effective in leishmaniasis are its efficacy along with relatively no or minimum side effects as compared to current therapeutic agents. The present study demonstrated that *C album* at a higher dose of 1000 mg/kg b. wt. not only reduced the parasite load but enhanced the DTH responses and did not cause any toxicity. Thus, it can be considered as a potential hepatoprotective and antileishmanial agent alone, or in combination therapy. However, future studies should be planned with higher doses and on higher animal models to come to a logical conclusion.

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COMPETING INTERESTS

Authors declare that no competing interests exist.

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