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Original Research Article (Experimental)

Protective efficacy of *Chlorophytum borivilianum* root extract against murine visceral leishmaniasis by immunomodulating the host responses

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ABSTRACT

Background: The available drugs for treating visceral leishmaniasis are limited. Moreover, the disease is associated with suppression of immune function. Therefore, therapies with effective immunomodulatory agents are needed which can decrease parasitic burden and enhance adaptive immunity.
Objectives: The present study was planned to evaluate the antileishmanial efficacy of crude ethanolic extract of roots of Chlorophytum borivilianum (CBREE) against murine visceral leishmaniasis through immunomodulation.
Materials and methods: The in vitro studies were carried out to check leishmanicidal activity against promastigote form and cytotoxicity against HeLa cells. The parasite load in liver smears, immunological and biochemical changes induced by 500 and 1000 mg/kg b.wt. of CBREE were assessed on 1, 7, 14 and 21 post treatment days in infected and treated BALB/c mice.
Results: CBREE showed inhibitory effect on growth of promastigotes with IC50 of 28.25 μg/mL and negligible cytotoxicity. The extract was toxicologically safe in BALB/c mice when administered orally with 5 g/kg b.wt. of extract. A significant reduction in parasite load was observed along with active immunomodulation through enhanced Th1 type of immune responses and suppressed Th2 type of immune responses.
Conclusion: The treatment with both doses showed no toxic effect as evidenced by normal liver and kidney function tests and normal histological observations of liver and kidney. Therefore, it should be further explored for its active components in pursuit of the new effective antileishmanial agents in the plant kingdom.

1. Introduction

Leishmaniasis represents a disease of grave concern especially in resource limited countries. Visceral leishmaniasis (VL) or kala azar, is the deadliest form amongst all forms of leishmaniasis which is endemic in developing and poor countries i.e. India, Brazil, Bangladesh, Sudan, South Sudan and Ethiopia. Approximately 2,00,000–4,00,000 new cases occur worldwide per year. In India itself, the disease is prevalent in poor states i.e. Uttar Pradesh, Bihar, Jharkhand and West Bengal. Out of total cases of VL worldwide, 90% of cases occur only in these states of India [1]. VL affects the visceral organs of host especially liver, spleen and lymph nodes. It also impairs the host defence system by altering host cell signalling pathways for their survival in phagocytic cells like macrophages and by modifying their cytokine and chemokine responses that lead to progression of disease within infected macrophages [2].

Treatment of leishmaniasis is a source of grave concern worldwide. Over the years, the treatment of leishmaniasis relies primarily upon few available drugs. The available drugs are associated with problems like long treatment duration, route of administration, toxicity and their variable efficacy [3,5]. Pentavalent antimony is a standard drug against leishmaniasis. In India, especially in Bihar, the resistance to antimonials has become a major challenge in the treatment of patients. A significant decrease in cure rate has also been observed in patients of Nepal living near the antimony resistant areas of Bihar [4]. The other second line drugs are

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amphotericin B and miltefosine. Amphotericin B suffers from various drawbacks like nephrotoxicity, nausea, vomiting, rigours, fever, hypertension/hypotension and hypoxia. In addition, it also causes hypokalaemia, hypomagnesaemia, metabolic acidaemia and polyuria [6]. The principle drawback of miltefosine is its teratogenicity and abortifacient ability, which completely abandoned its use in pregnant women. It also has a long half life which makes it vulnerable to resistance [7]. Liposomal amphotericin B is a new hope but more alternatives are needed for the effective treatment of VL, since the high cost of liposomal formulations make this drug unaffordable to the poor affected people.

Leishmaniasis is a disease which progresses with suppression of protective Th1 type of immune responses and up-regulation of non protective Th2 type of immune responses. Natural plant products and their phytochemical constituents are well known for their strong immunomodulatory properties [8]. Furthermore, 28% of the total drugs registered from 1981 to 2006 were either the plant products or their phytoconstituents [9]. Therefore, natural products with immunomodulatory properties can be potential source of new alternative compounds for treating neglected tropical diseases especially caused by protozoans like *Leishmania* [10]. The Bharia and other communities of Patalkot valley near Chindwara district of Madhya Pradesh employ *Chlorophytum borivilianum* i.e. safed musli as a native therapy for health care, particularly to improve immunity [11]. Hence, the current study focussed upon the anti-leishmanial efficacy of *C. borivilianum* was checked against VL since it has been reported to possess strong immunomodulatory [12], anthelmintic [13], antimicrobial [14], aphrodisiac and revitalizer [15] properties.

2. Materials and Methods

2.1. Promastigote culture

The MHOM/IN/80/Dd8 strain of *Leishmania donovani* was used for the present study. Log phase promastigotes were used for the maintenance of strain in modified NNN medium at 22 ± 1 °C. The culture was checked for any contamination and sub cultured after every 48–72 h by transferring 0.5–1.0 mL of culture suspension in Mc Cartney vials containing NNN medium and then supplemented with 3–4 mL of MEM. The pH of 7.2 of medium was regulated by adding 7.5% $NaHCO_3$. The promastigote culture was maintained in the B.O.D. incubator at 22 ± 1 °C [16].

2.2. Preparation of the extract

The *C. borivilianum* roots were purchased from the farmers of Karnal District of Haryana. The expert from the Department of Botany, Panjab University, Chandigarh has identified the plant and voucher no. 5328 was obtained by submitting the specimen. The roots were powdered after washing and air drying. The ethanolic extract was prepared by Soxhlet extraction method. Approximately 100 g dried and powdered roots were extracted with 250 mL of ethanol. The crude extract was filtered and filtrate was concentrated in rotary evaporator (Buchi, USA). The residue was then lyophilized and kept at −20 °C. It was equivalent to 6% of the dry mass of original rhizome powder.

2.2.1. Analysis of phytochemical constituents

The alkaloids, saponins, phenols, terpenes, flavonoids, glycosides, tannins and polysterols were detected through standard protocols [17,18].

2.2.2. GC–MS analysis of extract

The phytochemical investigation of ethanolic extract was performed on a GC–MS equipment i.e. Thermo Trace 1300 GC coupled with Thermo TSQ 800 Triple Quadrupole MS. The column used was TG 5MS (30 m × 0.25 mm, 0.25 μm). The flow rate of mobile phase was 1 mL/min. The oven temperature was 60 °C raised to 280 °C at the rate of 10 °C/min. The injection volume was 1.0 μL. Samples were dissolved in ethanol at a concentration of 1 mg/mL. The results were compared with NIST 2.0 library.

2.3. Parasite viability test

2×10^6 promastigotes were suspended in each well of 24 well culture plates. The specific concentrations (10–100 μg/mL) of plant extract were then poured into specifically marked treated wells. The test was performed in duplicate series. Control wells were treated with 1% DMSO and 10–100 μg/mL of sodium stibogluconate (SSG). The plates were kept at 22 ± 1 °C for 48 h. After incubation, promastigotes of *L. donovani* were stained with propidium iodide [19]. The percentage viability was calculated by probit analysis using SPSS 18.0 software.

2.4. Cytotoxicity test

The property of tetrazolium dye (MTT) to form blue formazan crystals in live cells was used to quantify the growth of HeLa cells. 6×10^3 cells were subsequently supplemented with specific concentrations (10–1000 μg/mL) of plant extracts. After 48 h of incubation, MTT (1 mg/mL) was added in each well by pipetting out earlier medium. After incubating the plates for three to 4 h, 100 μL of DMSO was added to each well. The absorbance was recorded at 560 nm. The effect of extract was expressed by CC_{50} values.

2.5. Acute toxicity

Limit test of Lorke [20] was used to estimate acute toxicity of plant extract. For this, inbred BALB/c mice were administered with different concentrations of extract by oral route. The extract was suspended in standard suspension vehicle i.e 5 g of carboxy methyl cellulose, 5 mL of $C_6H_5CH_2OH$ and 4 mL of Tween 80 in 1000 mL of 0.9% aqueous NaCl. 5 normal mice fasted for 4 h were then treated with upper dose limit of 5 g/kg b.wt. of extract. All the mice were observed for 14 days for antagonistic effects like mortality. Histopathological studies of liver and kidney were carried out at the end of study.

2.6. Groups of animals

The inbred BALB/c mice of 20–25 g weight, 4–6 weeks old of either sex were used for present study. They were kept in standard conditions of 25 ± 2 °C and fed with animal feed and water *ad libitum*. Promastigotes were harvested from 2 to 3 days old culture and 10^7 promastigotes were injected intracardially to mice [21]. Various groups included normal mice, infected mice, mice infected and treated with 40 mg/kg b.wt. of SSG for 5 days, mice infected and treated with 500 and 1000 mg/kg b.wt. of *C. borivilanum* roots ethanolic extract (CBREE) for 15 days after 30 post infection days. Each infected and treated group was divided into four subgroups and each subgroup comprised of six mice. Each subgroup was then sacrificed on 1, 7, 14 and 21 post treatment days (p.t.d.). The ethical clearance was obtained from Institutional Animal Ethics Committee (PU/IAEC/S/14/154) and experiments were conducted according to CPCSEA guidelines.

2.7. Assessment of parasite load

Giemsa stained liver imprint slides were studied and parasite load was calculated as Leishman Donovan Units (LDU) [22].

$$\text{LDU} = \frac{\text{No.of amastigotes}}{\text{No.of liver cell nuclei}} \times \text{Weight of organ (in mg)}$$

2.8. Determination of immune responses by

2.8.1. Cell mediated immune responses

2.8.1.1. Cytokine responses. The IL-12, IL-4, IL-10 and IFN-γ levels were assayed by ELISA in serum samples of mice using Diaclone kits.

2.8.1.2. Delayed type hypersensitivity (DTH) responses. Leishmanin antigen was injected subcutaneously into right foot pad of mice of all groups and left foot pad with phosphate buffer saline. After 48 h, the swellings of both foot pads were measured using a pair of vernier callipers and percentage increase in thickness of the right foot pad as compared to the left was calculated [21].

2.8.2. Humoral immune responses

The levels of immunoglobulins IgG1 and IgG2a were calculated through ELISA [21]. ELISA plates were coated with 10 μg *L. donovani* crude antigen. Sera of mice from different groups were diluted two fold by serial dilutions and added to specific wells. Isotype specific HRP-conjugated secondary antibodies (rabbit antimouse IgG1 or IgG2a) were then added. Substrate 3,3′,4,4′-Tetramethylbenzidine was added in order to develop colour and absorbance was taken on ELISA plate reader at 450 nm.

2.9. Liver and kidney function tests

The serum levels of glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), acid phosphatase (ACP), lactate dehydrogenase (LDH), urea, creatinine and uric acid were measured by Thermo Fisher Scientific India Pvt. Ltd. kits.

2.10. Histopathological parameters

Kidney and liver tissues from each subgroup were sectioned and stained with haematoxylin and eosin (HE) to study histology.

2.11. Statistical analysis

The results were calculated as mean ± S.D. The significance between mean values of two groups were analysed by one way ANOVA using Tukey post hoc test. P values less than 0.05 were considered as significant.

3. Results

The preliminary phytochemical screening of ethanolic extract of roots of *C. borivilianum* revealed alkaloids, flavonoids, saponins, diterpenes, triterpenes, polysterols, phenols, tannins and glycosides (Table 1). The GC–MS analysis of ethanolic extract of this plant lead to the identification of a number of compounds (Table 2, Fig. 1).

3.1. In vitro antileishmanial activity of C. borivilianum roots ethanolic extract

The promastigotes of *L. donovani* were treated with various doses of CBREE. The growth inhibition of promastigotes was in dose dependent manner. The maximum growth inhibition was observed to be 78.19% at 100 μg/mL, whereas minimum inhibition of 41% was found at 10 μg/mL of CBREE. The IC50 was calculated by plotting dose response curve by using SPSS and was found to be 28.53 μg/mL. Whereas, the IC50 of SSG was calculated 30.15 μg/mL (Fig. 2).

3.2. Cytotoxic effect of C. borivilianum roots ethanolic extract

The CBREE and SSG exhibited no toxicity towards HeLa cells. The CC_{50} was determined and found to be more than 1000 μg/mL for CBREE. The percentage cell viability was found to be 78.07 ± 1.33% at the highest concentration of 1000 μg/mL and 72.58 ± 0.89% at the lowest concentration of 100 μg/mL of CBREE.

3.3. Selectivity index of C. borivilianum roots ethanolic extract

Selectivity index was calculated as ratio of cytotoxic effect of extract on HeLa cells to growth inhibition of extract against parasites. The value of selectivity index greater than 10 was considered to have good antileishmanial activity. The CBREE was found to possess the selectivity index of 35.05.

3.4. Acute toxicity

The CBREE was found tolerant and safe at the higher dose of 5 g/kg b.wt. as all the mice were active during 14 days observation period. No changes like restlessness, sluggishness and abdominal contractions were shown by mice within 2 h of administration of CBREE. Histological examination of liver and kidney did not reveal any signs of hepatomegaly and renal toxicity and were well comparable to the normal mice. Morphology of both liver and kidney were also normal.

3.5. In vivo antileishmanial activity of C. borivilianum roots ethanolic extract

Treatment of animals with CBREE significantly ($p < 0.05$) decreased the parasite load when compared with infected animals. The animals treated with higher dose of CBREE showed least parasite load ($p < 0.05$) and the parasite load lessened by 94.71% (224.1 ± 20.47) on 21 p.t.d. Significant reduction of 92.09% in parasite burden was also observed in animals treated with SSG after 21 p.t.d. The decrease in parasite load in animals treated with higher dose of CBREE was comparable to animals treated with SSG (Fig. 3).

3.5.1. Th1 type immune responses

The cytokines IFN-γ and IL-12 was found to be high in the treated animals as compared to the only infected animals. A massive upregulation of these cytokines was found in animals treated with CBREE ($p < 0.05$). Maximum levels were measured in animals treated with higher dose of CBREE followed by animals treated with lower dose of CBREE. Animals treated with SSG produced lesser cytokine levels ($p < 0.05$) as compared to CBREE treated groups (Fig. 4A and B).

3.5.2. Th2 type immune responses

IL-4 and IL-10 cytokines are considered to be signature cytokines of the Th2 type of immune responses and their levels were significantly ($p < 0.05$) lower in treated animals in comparison to

Table 1
Phytochemical constituents of *C. borivilianum* ethanolic extract.

Phytochemical constituents	Tests/reagents	Result
Alkaloids	Wagner's test/Hager's test	+
Flavonoids	Alkaline reagent test/Lead acetate test	+
Saponins	Froth test/Foam test	+
Terpenes	Copper acetate test	+
Polysterols	Salkowski's Test/Libermann Burchard's test	+
Phenols	Ferric chloride test	+
Tannins	Gelatin test	+
Anthranol glycosides	Modified Borntrager's test	−

Table 2
Various compounds of ethanolic extract of roots of *C. borivilianum*.

Compound name	Retention time	Molecular formula	Peak area
1,4,7,10,13,16-Hexaoxacyclooctadecane	6.17	$C_{12}H_{24}O_6$	1.87
Ethyl 14-hydroxy-3,6,9,12-tetraoxatetradecan-1-oate	6.67	$C_{12}H_{24}O_7$	23.94
Butyl 23-hydroxy-3,6,9,12,15,18,21-heptaoxatricosan-1-oate	7.03	$C_{20}H_{40}O_{10}$	1.25
Propane, 1,1-diethoxy-2-methyl	7.45	$C_8H_{18}O_2$	6.45
Camphor	8.17	$C_{10}H_{16}O$	8.83
1,7,7-Trimethylbicyclo[2.2.1]heptan-2-ol	8.39	$C_{10}H_{18}O$	2.14
Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)1	11.69	$C_{15}H_{24}$	1.42
Lanceol, cis	12.14	$C_{15}H_{24}O$	0.40
Naphthalene	12.96	$C_{15}H_{24}$	0.30
Cycloisolongifolene, 8,9-dihydro-9-formyl	14.36	$C_{16}H_{22}O$	7.11
9-Isopropyl1-1-methyl-2-methylene-5-oxatricyclo[5.4.0.0(3,8)]undecane	14.66	$C_{15}H_{24}O$	0.71
3,7-Cyclodecadien-1-one,10-(1-methylethenyl)-(E,E)-	15.45	$C_{13}H_{18}O$	1.87
D-Norandrostane (5a,14a)	15.72	$C_{18}H_{30}$	0.47
Formic acid, 2-bromomethyl-4,4-dimethyl-3-(3-oxobut-1-enyl)cyclohex-2-enyl ester	15.84	$C_{14}H_{19}BrO_3$	1.48
Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-,hydrazide	17.89	$C_{18}H_{28}N_2O_2$	0.44
Hexadecanoic acid, 2-methyl-, methyl ester	18.31	$C_{18}H_{36}O_2$	0.34
9-Octadecanoic acid (Z)-, methyl ester	19.93	$C_{19}H_{36}O_2$	1.30

only infected animals. Only infected animals showed highest levels of these cytokines, however significant reduction ($p < 0.05$) was measured in treated animals. The cytokine levels were lowest in animals treated with higher dose of CBREE followed by lower dose of CBREE. Animals treated with SSG also produced lower levels of these cytokines in comparison to the infected controls ($p < 0.05$). The levels of IL-4 were significantly ($p < 0.05$) lower in animals treated with both the doses of CBREE as compared to SSG treated animals (Fig. 4C and D).

3.5.3. Delayed type hypersensitivity (DTH) responses

The DTH response was significantly increased in animals treated with CBREE in comparison to the infected controls ($p < 0.05$). Mice treated with higher dose of CBREE induced the highest DTH responses as compared to those animals treated with lower dose of CBREE and SSG. However, when both higher and lower doses of CBREE groups were compared with each other, the difference was significant ($p < 0.05$) on all post treatment days. A significant rise

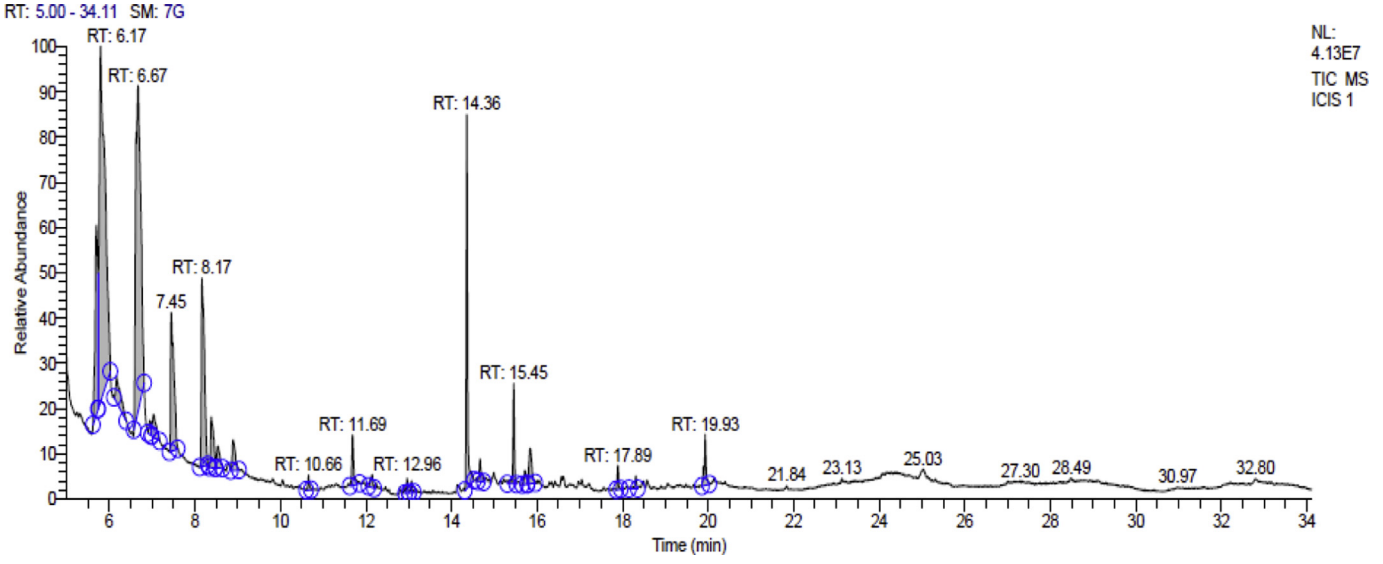

Fig. 1. GC-MS chromatogram of crude ethanolic extract of roots of *C. borivilianum*.

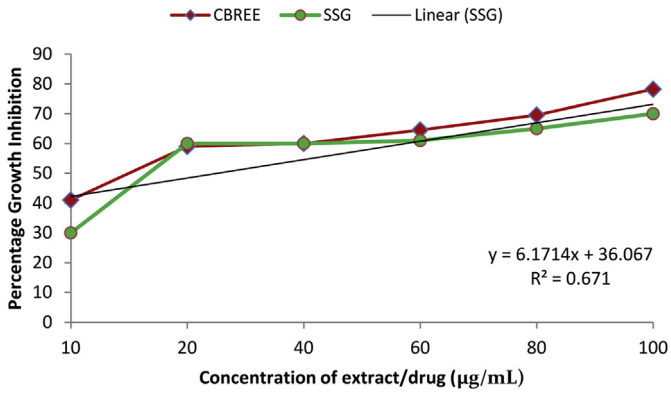

Fig. 2. Percentage growth inhibition of *L. donovani* promastigotes with C. *borivilianum* ethanolic extract.

($p < 0.05$) was also found when SSG treated animals were compared with infected group (Fig. 5).

3.6. Humoral immune responses

The IgG1 antibody levels, represent Th2 type (non protective type) of immune response, were found to be significantly reduced in CBREE treated animals as compared to the infected controls ($p < 0.05$). Minimum antibody levels were detected in animals treated with higher dose of CBREE on all p.t.d. Animals treated with SSG also produced lower antibody titres in comparison to the infected controls ($p < 0.05$) (Fig. 6A).

The IgG2a antibody levels represent protective Th1 type of immune responses. Maximum amount of IgG2a antibody was produced by CBREE treated animals. Increase in antibody levels was also observed in animals treated with SSG. However, the levels were significantly ($p < 0.05$) lesser than CBREE treated groups (Fig. 6B).

3.7. Liver function tests

The levels of SGOT and SGPT in animals treated with SSG were found to be above the normal range on all post treatment days. They were significantly higher as compared to the normal controls ($p < 0.05$) but when compared with infected controls, the levels were not significant ($p > 0.05$) on 7, 14 and 21 p.t.d. When the animals were treated with 1000 mg/kg b.wt. of CBREE enzymatic activity was found to be similar to the normal controls ($p > 0.05$) and within the normal range on all post treatment days. Similarly,

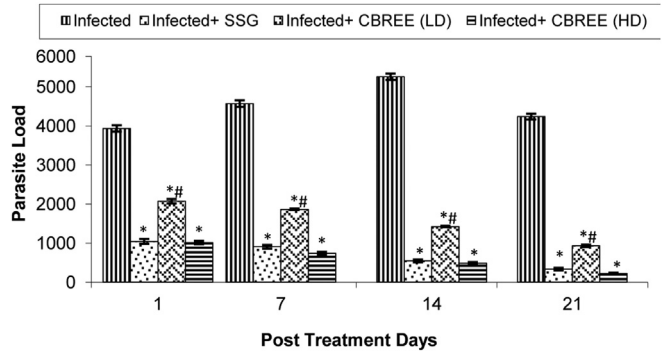

Fig. 3. Parasite load in different groups of animals P value: Infected + SSG, infected + CBREE (LD), infected + CBREE (HD) compared with infected control *$p < 0.05$ Infected + CBREE (LD), infected + CBREE (HD) compared with infected + SSG #$p < 0.05$.

in animals treated with 500 mg/kg b.wt. of CBREE, the levels were observed to be within the normal range on all post treatment days. The activities of ALP, ACP and LDH were found to be within the normal ranges in all the groups of animals (Table 3).

3.8. Kidney function tests

The blood urea concentrations were significantly ($p < 0.05$) higher in infected mice as compared to normal controls on 1 p.t.d. The concentrations were also significantly higher on 7, 14 and 21 p.t.d. in infected animals but were within the normal range of 10–45 mg/dL. Further, a significant decline ($p < 0.05$) in urea concentration was seen in animals treated with SSG when compared with the infected controls and was within the normal range. Moreover it was observed that in all the CBREE treated animals the urea concentrations were found to be non significant ($p > 0.05$) on 1, 7, 14 and 21 p.t.d when compared to the normal controls and were within the normal range. The levels of creatinine and uric acid were in normal range in all the groups of animals (Table 3).

3.9. Histopathological studies

Light microscopic examination of liver of infected mice showed kupffer cell hyperplasia as well as vacuolisation of hepatocytes (Fig. 7B). However, the normal architecture was retained when mice were treated with both the doses of plant extract (Fig. 7C and D). The transverse sections of kidney of infected mice revealed the lymphocytic infiltration at several places along with compressed glomerulus (Fig. 8B). The glomerulus, PCT, DCT and collecting tubules were found to be in normal appearance in mice treated with both the doses (Fig. 8C and D).

4. Discussion

C. borivilianum Santapau & Fern., belonging to family liliaceae is an important traditionally used plant in India, also known as 'white gold' or 'divya aushad'. In Ayurvedic system of medicine, it is known for its rejuvenating, natural sex tonic properties and reducing sexual dysfunctions [11]. The various phytochemical constituents such as saponins, alkaloids, flavonoids, terpenoids, steroids, gallotanins, phenolic acids, fructans and fructooligosaccharides (FOS), acetylated mannans and proteins have been revealed from its roots [13,23–27]. In the present study also, the major phytochemicals found were saponins, alkaloids, flavonoids, terpenes, phytosterols, tannins and other phenolic components. The GC–MS analysis of the extract also revealed the presence of various phytochemical constituents.

The preliminary experiments were carried out to examine the antileishmanial activity of crude ethanolic extract against promastigotes in *in vitro* conditions. It has been found that the antileishmanial activity of extract was better than the standard drug SSG. The IC50 was found to be 28.53 µg/mL and 30.15 µg/mL for extract and SSG respectively. Whereas both the extract and SSG exhibited negligible to no cytotoxicity against HeLa cells. Therefore, the selectivity index was found to be 35.05 for CBREE. The value of selectivity index for any extract greater than 10 is considered to have good antileishmanial activity which encouraged us to further explore the antileishmanial activity of this extract in animal model i.e. inbred BALB/c mice. Several studies have shown that the saponins, terpenes [28,29], alkaloids [30,31], flavonoids [32,33] and phytosterols are phytochemical constituents which possess antileishmanial activities. The antileishmanial efficacy of this plant can be accredited to any of these phytochemicals.

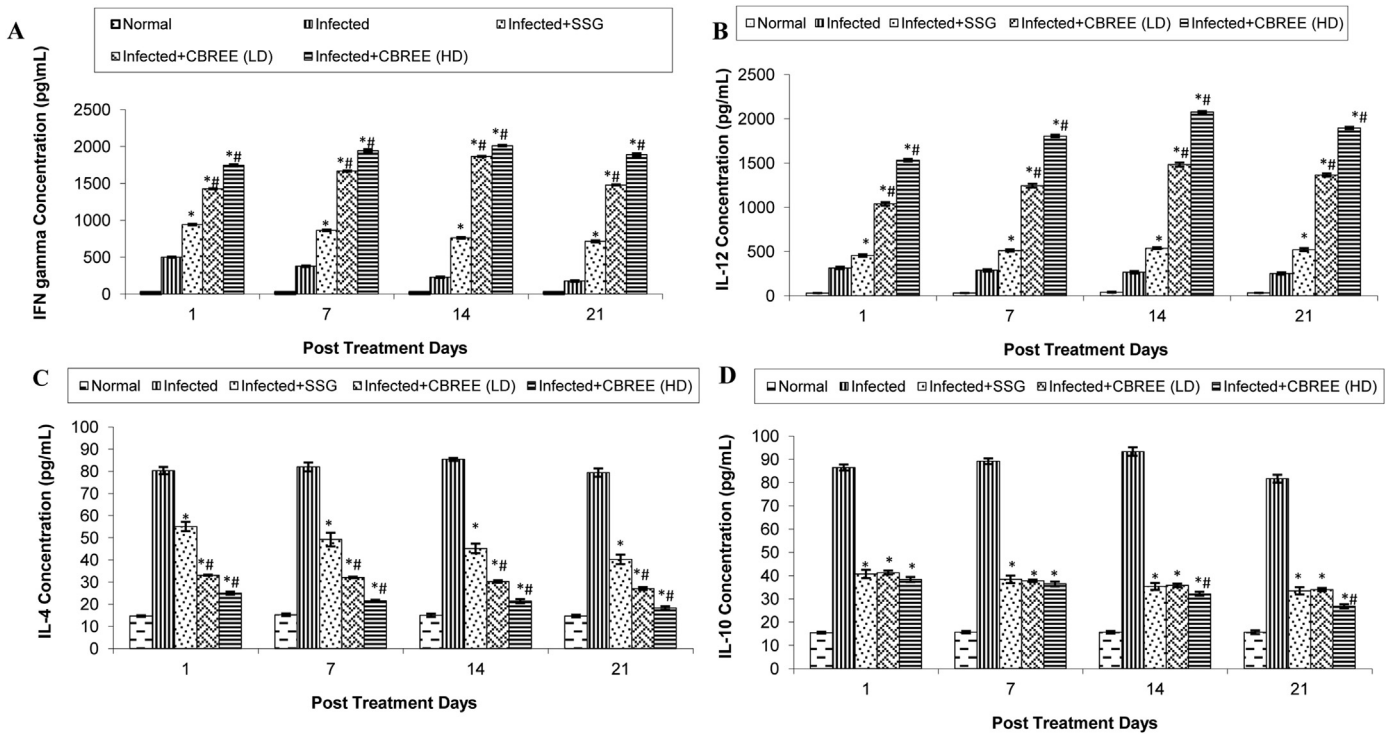

Fig. 4. A–D Concentration of cytokines IFNγ, IL-12, IL-4, IL-10 in serum samples of different groups of animal. P value: Infected+SSG, infected+CBREE (LD), infected+CBREE (HD) compared with infected control *p < 0.05 Infected+CBREE (LD), infected+CBREE (HD) compared with infected + SSG #p < 0.05.

The antileishmanial efficacy can also be attributed to the immunomodulatory potential of this plant extract. There is generalised immunosuppression in humans and animals during VL. Resolution of *Leishmania* infection depends upon the coordinated interaction between different constituents of cellular immune responses specially the sensitisation of T helper cells to produce specific cytokines and to activate macrophages for phagocytosis of parasite [34]. Therefore, any drug or plant extract which can retrieve the protective Th1 type of immune responses in hosts could be a good alternative against standard drugs. The ethanolic extract of *C. borivilianum* has been found to enhance both the cellular and humoral immune responses. The Th1 type of immune responses were observed as show by enhanced levels of IFN-γ, IL-12 cytokines and delayed type hypersensitivity responses which helps in providing significant protection against VL. Whereas, the Th2 type of immune responses i.e. levels of IL-10 and IL-4 cytokines which helps in the progression of disease were significantly decreased in mice treated with both the doses of CBREE. Our results

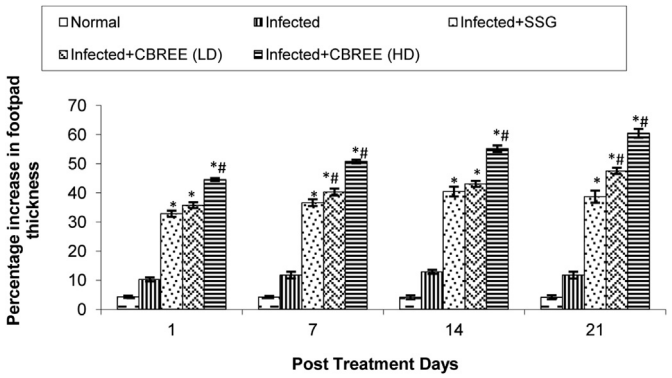

Fig. 5. Delayed type hypersensitivity response in animals of different groups. P value: Infected + SSG, infected + CBREE (LD), infected + CBREE (HD) compared with infected control *p < 0.05. Infected + CBREE (LD), infected + CBREE (HD) compared with infected + SSG #p < 0.05.

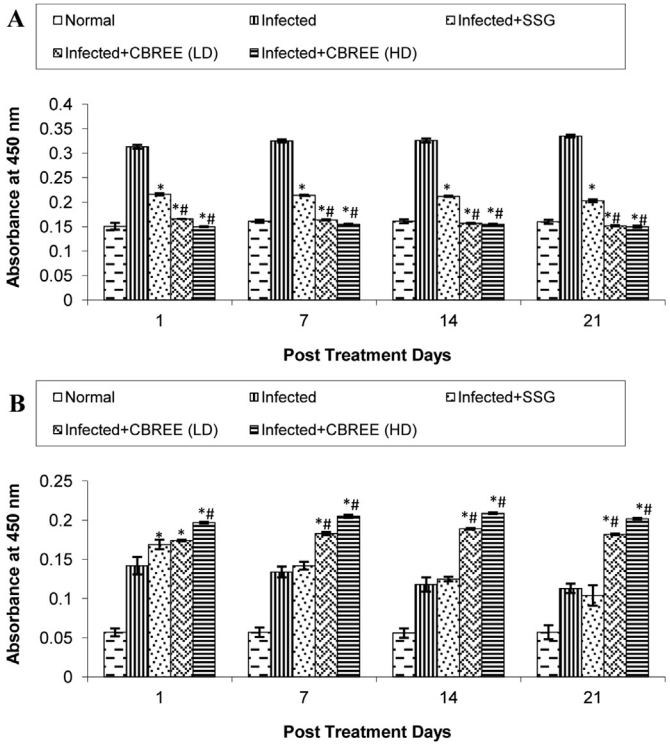

Fig. 6. A, B Levels of IgG2a and IgG1 antibodies in different groups of animals. P value: Infected + SSG, infected + CBREE (LD), infected + CBREE (HD) compared with infected control *p < 0.05. Infected + CBREE (LD), infected + CBREE (HD) compared with infected + SSG #p < 0.05.

Table 3
Acitivities of enzymes and blood urea levels in different groups of animals P value: Infected + SSG, infected + CBREE (LD), infected+CBREE (HD) compared with infected control *p < 0.05.

Enzymes (normal range)	PT days	Normal control	Infected control	SSG (40 mg/kg b.wt.)	Infected + SSG (40 mg/kg b.wt.)	Infected + CBREE (500 mg/kg b.wt.)	Infected + CBREE (1000 mg/kg b.wt.)
SGOT (5–40 IU/L)	1	25.33 ± 2.02	48.36 ± 1.04	42.89 ± 2.17	60.36 ± 2.74*	32.15 ± 1.87*	31.2 ± 1.64*
	7	26.89 ± 2.44	51.10 ± 2.01	30.67 ± 1.68	54.84 ± 2.7	32.02 ± 2.07*	30.51 ± 2.43*
	14	28.13 ± 4.47	53.27 ± 2.57	28.91 ± 2.2	54.48 ± 2.59	26.14 ± 2.03*	25.66 ± 2.4*
	21	23.53 ± 4.19	49.45 ± 3.16	29.19 ± 1.29	47.19 ± 2.33	27.63 ± 3.59*	22.81 ± 2.12*
SGPT (5–35 IU/L)	1	22.25 ± 2.16	69.63 ± 7.03	60.64 ± 6.89	96.85 ± 9.68*	23.67 ± 3.06*	25.23 ± 2.43*
	7	24.18 ± 1.94	80.65 ± 8.96	37.87 ± 4.16	84.57 ± 6.93	25.99 ± 2.37*	26.37 ± 1.81*
	14	23.97 ± 4.64	85.51 ± 5.93	33.28 ± 8.87	88.76 ± 9.28	23.67 ± 1.97*	23.91 ± 2.3*
	21	24.75 ± 5.03	76.69 ± 5.71	30.49 ± 5.46#	85.86 ± 6.5	23.77 ± 1.74*	25.17 ± 2.1*
UREA (10-45 mg/dl)	1	24.25 ± 1.77	49.98 ± 2.29	35.15 ± 2.23	34.73 ± 2.89*	23.45 ± 2.17*	22.45 ± 1.75*
	7	24.62 ± 2.68	44.84 ± 3.05	32.47 ± 2.17	34.1 ± 3.02*	25.51 ± 1.37*	23.78 ± 2.09*
	14	22.66 ± 2.5	42.96 ± 3.39	29.36 ± 0.69	34.31 ± 1.53*	25.85 ± 1.72*	25.18 ± 1.33*
	21	23.29 ± 2.59	44.73 ± 2.89	31.43 ± 0.84	34.02 ± 2.17*	22.54 ± 3.22*	24.89 ± 2.67*

Signifies p value is less than 0.05 when animals treated with higher dose and lower dose are compared with animals treated with standard drug.

Table 4
Serum levels of urea in various groups of animals. P value: Infected + SSG, infected + CBREE (LD), infected + CBREE (HD) compared with infected control *p < 0.05.

Enzymes (normal range)	PT days	Normal control	Infected control	SSG (40 mg/kg b.wt.)	Infected + SSG (40 mg/kg b.wt.)	Infected + CBREE (500 mg/kg b.wt.)	Infected + CBREE (1000 mg/kg b.wt.)
UREA (10–45 mg/dl)	1	24.25 ± 1.77	49.98 ± 2.29	35.15 ± 2.23	34.73 ± 2.89*	23.45 ± 2.17*	22.45 ± 1.75*
	7	24.62 ± 2.68	44.84 ± 3.05	32.47 ± 2.17	34.1 ± 3.02*	25.51 ± 1.37*	23.78 ± 2.09*
	14	22.66 ± 2.5	42.96 ± 3.39	29.36 ± 0.69	34.31 ± 1.53*	25.85 ± 1.72*	25.18 ± 1.33*
	21	23.29 ± 2.59	44.73 ± 2.89	31.43 ± 0.84	34.02 ± 2.17*	22.54 ± 3.22*	24.89 ± 2.67*

were also supported by other studies. The polysaccharide fractions isolated from hot water extract of this plant have also enhanced the NK cells activity and humoral responses to sheep RBCs in Wistar rats [35]. Likewise, the ethanolic extract of root tubers of this plant exhibited strong immunomodulatory activity against *Candida albicans* by enhancing DTH responses, neutrophil adhesion and phagocytosis of fungal hyphae [12]. The aqueous extract of this plant has also increased the concentration of IFN-γ cytokine level at the dosage of 250 and 500 mg/kg b.wt. in wistar rats against viral infection [36]. In addition, several studies have shown that the plant extracts with immunomodulatory properties possess significant antileishmanial activity. The semi purified leaf extract of *Croton caudatus* has been demonstrated to possess antileishmanial efficacy against both amastigote and promastigote stage of *L. donovani* by enhancing the levels of IFNγ and suppressing the levels of IL10 thus modulating the immune responses [37]. In

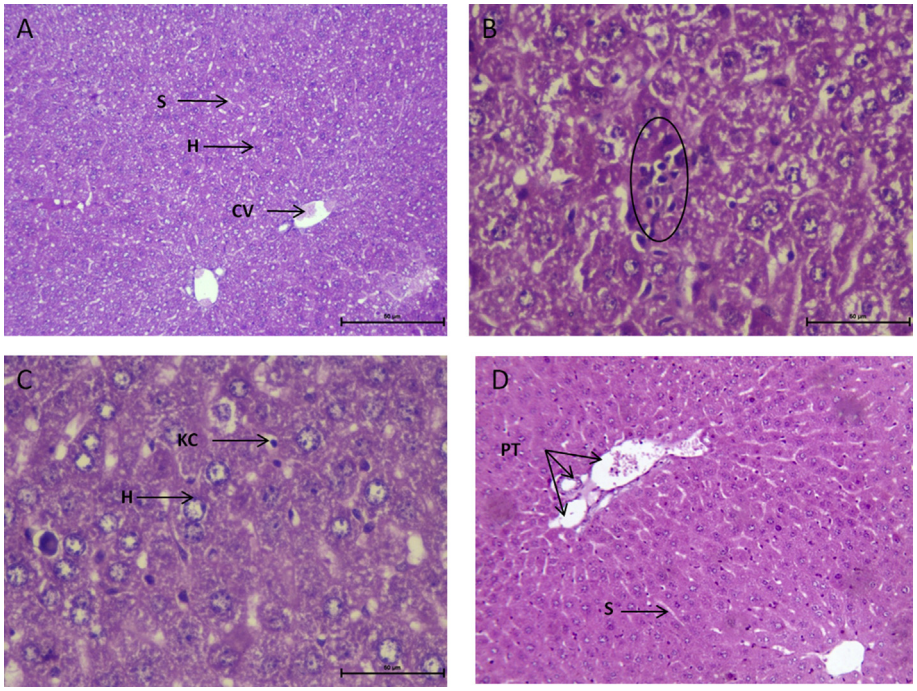

Fig. 7. T.S. of liver of BALB/c mice stained with haematoxylin and eosin stain (A) Normal control (10×) (B) Infected Control (40×) (C) Infected mice treated with CBREE (LD) 10× (D) Mice treated with CBREE (HD) 10×.

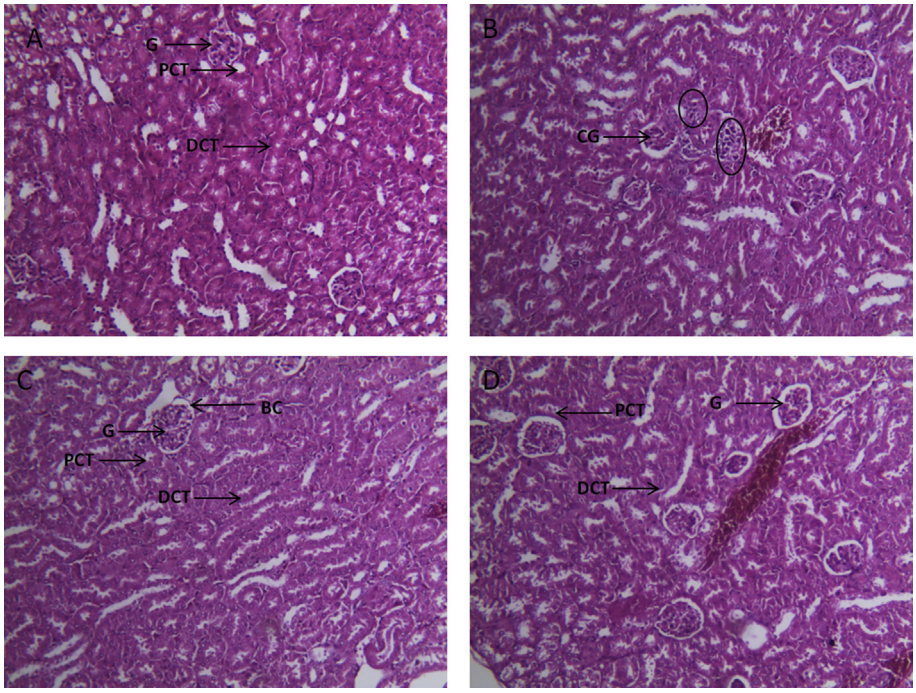

Fig. 8. T.S. of kidney of BALB/c mice stained with haematoxylin and eosin stain (A) Normal control (10×) (B) Infected Control (10×) (C) Infected mice treated with CBREE (LD) 10× (D) Mice treated with CBREE (HD) 40×.

another study, the ethyl acetate extract of neem has been shown to possess inhibitory effect on promastigotes by upregulating the Th1 cytokine responses which lead to disease suppression [38].

Humoral response to leishmaniasis is characterized by the presence of antileishmanial antibodies in blood. These antibodies are produced in very less quantity in CL but at very high level in VL. But their antileishmanial role against VL is not very clear [39]. In the present study also, significant rise in IgG2a antibody levels and significant decline in IgG1 levels was detected in all the treated groups of animals.

The treatment of animals with SSG has shown significant cure but the drug has been found to be associated with arthralgia, hepatitis, pancreatitis and cardiac dysrhythmias [40]. The increased levels of serum amylase and alanine transaminase have been reported in cutaneous leishmaniasis patients when treated with SSG [5]. In the present study also, the animals treated with SSG showed significantly elevated levels of SGOT and SGPT as compared to normal controls. However, the mice treated with both the lower and higher doses of CBREE showed normal levels of all the liver function tests. The hepatoprotective nature of CBREE has also been reported against arsenic induced toxicity with normal histopathology of liver and normal levels of liver enzymes [41]. Similarly, the histology of liver and kidney tissue of mice treated with both the doses of extract has been found to be normal in the present study.

As it is mentioned above that a majority of the treatment regimens against leishmaniasis have severe adverse reactions that decrease patient's consent with treatment regimes, and in some patients, the treatment has to be stopped inbetween. In addition, tropical diseases like leishmaniasis are mainly ignored in drug discovery programs by most of the pharmaceutical companies because of inadequacy of sufficient funds for this costly and laborious process [42]. On the other hand, medicinal and aromatic plants are main sources of natural organic compounds which have been enormously used as medicine. These plants are clearly a potential source of new antileishmanial drugs. Large number of natural plant products and lead compounds are derived from plants and microbes. Therefore, the extract or secondary metabolites found in this plant, which can improve the pathological and immunological conditions, can be seen as attractive candidates for the development of new therapeutics against leishmaniasis.

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Conflicts of interest

None.

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