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Research article

# Antileishmanial Effect and Immunomodulatory Activity of *Tinospora cordifolia* and *Withania somnifera* against Experimental BALB/c Mice

**Ritesh Kumar Tiwari[1], Gaurav Kumar Pandit[1], Ashish Kumar[2], Kundan Kunal[2], Veer Singh[2], Ghufran Ahmed[2], Meenakshi Singh[1*]**

[1]*Department of Botany, Patna University, Patna-800005, Bihar, India.*
[2]*Department of Biochemistry, ICMR-Rajendra Memorial Research Institute of Medical Sciences, Patna-800007, Bihar, India*

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ABSTRACT

*Visceral leishmaniasis* (VL) is a vector-borne disease that progresses mainly by lowering of immune protective cells Th1 and the appearance of cells Th2 that promote illness; therefore, the treatment of this disease relies on improving the immune condition of the host. Currently used treatment options for VL are Amphotericin B (AmB) and its liposomal formulation, i.e., Ambisome. But these treatment options are not safe as these drugs have many side effects and limitations like hepatotoxicity, nephrotoxicity, VL relapse and PKDL conversion. This has prompted the search for alternative treatment options amongst herbal drugs; Thus, this study investigated *Tinospora cordifolia* and *Withania somnifera's* antileishmanial potency, either in combination or alone. In vivo experiments demonstrated a substantial decrease in BALB/c mice in the spleen parasitic burden. Moreover, the treatment effectively modulated the immune response of the host, leading to Th1 polarization, crucial for eliminating *Leishmania donovani*. This method not only targets the parasite but also fortifies the immune system, offering a safer, cost-effective alternative to current therapies for *V. leishmaniasis*, which are often limited by toxicity and resistance.

## Introduction

*Visceral leishmaniasis* (VL), alternatively referred to as 'kala-azar', is attributed to the obligatory intracellular protozoan parasite *Leishmania donovani* in South Asia and Africa.[1] The characteristic indications of VL include intermittent pyrexia, enlarged liver and spleen, a decrease in blood cells and weight loss. This disease can be cured in most cases if treated, but if left untreated, it can worsen and become fatal.[2,3] Annually, there are about 2,00,000 to 4,00,000 VL incidences, out of which almost 90% of these occur in poor, rural areas of six countries: India, Bangladesh, Ethiopia, Brazil, Sudan, and South Sudan.[4] In India, *V. leishmaniasis* is especially prevalent in states like Bihar, Uttar Pradesh, Jharkhand, & West Bengal. In fact, approximately 90% of *V. leishmaniasis* cases in India are reported solely from Bihar.[5] One of the main characteristics of VL is immunosuppression, which manifests as a lowered Thl response and an increased Th2 response at the same time.[5] Resolution and resistance to *V. leishmaniasis* are linked to Thl cell-mediated immune responses, while Th2 immune responses increase vulnerability. Th1 cells secrete pro-inflammatory cytokines for example, IFN-γ, TNF-α, IL-2, and IL-12, while Th2 cells predominantly discharge anti-inflammatory cytokines comprising IL-4, IL-10, and IL-13. [5,6] IFN-γ and IL-12 activate macrophages, leading to the production of nitric oxide (NO), hence enhancing resistance to infection..[7,8]

***Corresponding Author:** Prof. Meenakshi Singh

**Address:** Department of Botany, Patna University, Patna-800005, Bihar, India.

**Email** ✉**:** meenakshisingh.pu@gmail.com

**Tel.:** 91-9006645622

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A significant immunosuppressor cytokine, IL-10, is crucial for the sustenance of parasites and contributes significantly to the worsening of the illness.[9] Thus, immune response skewing regimens that change the dominant disease-promoting immune response to Thl-mediated protection may consequently promote healing in addition to the leishmanicidal effects of the medications.[10] In India, the currently used treatment options for VL are Amphotericin B (AmB) and its liposomal formulation, i.e., AmBisome.[11] But the treatment approach of VL with these drugs also has many side effects and limitations, like hepatotoxicity, nephrotoxicity, VL relapse, and PKDL conversion.[12,13] Nevertheless, only some effective medicines are available and each has a particular disadvantage and limitations, such as toxicity and teratogenicity; long-term regimens compromise many chemotherapeutic options.[14] We must therefore adopt a safer, more efficient and more cost-effective approach for treating VL. Traditional medicinal plants serve the purpose of being safe because a significant portion of the global population uses traditional medicine, and ecological evidence also suggests that herbal products are considered harmless by folk or traditional healers.[15–17] Among traditionally available medicinal plants, *Tinospora cordifolia* (Tc) and *Withania somnifera* (Ws) and were the most suitable candidates because of their wide acceptance as traditional medicinal plants for fighting against the disease. Based on the spatial distribution of both plants, chemo-typical Variations are likely to occur in these plants, some of which may be genotypical in nature.[18,19] Consequently, a study of pharmacological and immunomodulatory activity is urgently required against VL. Consequently, this research aimed to examine the synergistic impact of *T. cordifolia* (Tc) and *W. somnifera* (Ws) as immunotherapeutic agents and to compare their antileishmanial efficacy with standard available antileishmanial drug AmB or AmBisome.

This study focuses on the evaluation of the anti-leishmanial potential of Tc and Ws individually and combined on *in-vivo* VL BALB/c mice in reference to AmB or AmBisome treatment. Further, the experiments of biochemical parameters were performed to evaluate the immunological condition of the host and toxicities (liver and kidney).

## Materials and Methods

### Leishmania Parasite Culture

The promastigotes form of *Leishmania donovani* (Strains: MHOM/IN/1983/AG83) parasites were obtained from the cryobank collection of the ICMR-RMRIMS. Parasite culture was performed in RPMI-1640 medium (Sigma-Aldrich), enriched at 10% heat-inactivated fetal bovine serum (HIFBS), and antibiotics like 25 µg/mL gentamicin, 50 µg/mL streptomycin & 50 U/mL penicillin (Sigma-Aldrich). Parasites were incubated in biological oxygen demand (BOD) at 24°C for growth, and once enough growth occurred in 3 to 4 days and the culture reached the stationary stage, the promastigotes were subcultured into fresh RPMI medium in T-25 flasks (NuncTM). The infective promastigote stage of *Leishmania* parasite (metacyclic form) was prepared and harvested by the Ficoll gradient method as detailed.[20]

### Ethical Committee

BALB/c animals were acquired from the animal establishment of ICMR-RMRIMS Patna. Female mice belonging to 6-8 weeks old and weighing between 25 to 30 g were chosen for this experiment. The animals selected were kept healthy by regular checkups and being provided with sustenance and hydration *ad libitum*. The experimental mice were housed under optimal conditions, particularly, an average temperature of 25 ± 2°C, a photoperiod of 12 hours of night and 12 hours of daylight, and a relative humidity of 55 ± 10%. All animal studies were conducted after approval from the Institutional Animal Ethics Committee (IAEC) of RMRIMS (letter No. RMRI/EC/008/2021). The experimental animals received an intraperitoneal anesthetic combination of ketamine (5 mg/kg) and xylazine (4 mg/kg).[21] Additional mice were euthanized via cervical dislocation. All procedures, including injections, blood collection and euthanasia, followed the ethical guidelines set forth by the IAEC and CPCSEA, Government of India.

### Drugs

Tc and Ws were purchased as pure herbs in the form of tablets (of whole plant) from the Himalaya Drugs company, Bangalore, India. These pure herbal extracts were solubilized in different solvents like ethanol, methanol, distilled water, and DMSO to get the required drug concentration of mg $mL^{-1}$. The dissolved drug was further diluted with the appropriate solvent before experimenting. The standard antileishmanial drug Amphotericin B was acquired from Sigma-Aldrich. AmBisome used in the experiment was obtained from ICMR-RMRIMS, Patna.

### *In-vivo* Infection and Treatment

Inbred BALB/c animal were subjected to intracardiac infection with $10^7$ promastigotes of *L. donovani* as per the protocol described in previous studies.[22] The infected mice were administered 30 days after infection, via the abdomen, with different groups containing Tc and Ws. The effectiveness of Tc + Ws extract was calculated by observing the extent of *Leishmania* parasite clearance in the VL model.[20] For this experiment, 6 to 8-week-old inbred BALB/c mice were selected and categorized into seven groups with six animals in every group (Group I–VII).

Group I: Control (Only PBS)
Group II: *Leishmania* infection
Group III: *T. cordifolia* (Tc)

Group IV: *W. somnifera* (Ws)
Group V: Pure AmB
Group VI: AmBisome
Group VII: Tc-Ws (single dose)
Group VIII: Tc-Ws (double dose)

### Assessment of Infection

To confirm infection, two randomly selected mice were evaluated six weeks after infection. After confirmation, the infected mice were assigned to different treatment groups and administered the respective treatments intraperitoneally. After two weeks of treatment, these mice were euthanized and the presence of parasites was assessed by Giemsa staining of splenic imprints. The parasitic load was quantified Microscopically by enumerating the amastigotes present in 1000 fields.[15,20]

### Extracellular Cytokines Estimation by ELISA

Splenocytes were freshly isolated from each group of experimental mice and seeded into 12-well culture plates. The cells were then stimulated with appropriate concentrations of Tc and Ws extracts and incubated at 37°C in a $CO_2$ incubator (5% $CO_2$, 95% humidity) for 48 hours. Following incubation, the cell-free supernatant was collected by centrifuging the culture at 700 g for 15 minutes at room temperature (24 ± 1°C). The levels of cytokines—including IFN-γ, IL-12, and IL-10—were subsequently quantified using commercially available ELISA kits (BD OptEIA™, ELISA Kit), in accordance with the manufacturer's instructions. [6]

### Biochemical Analysis

Blood Samples were gathered from every group of BALB/c animals via retro-orbital/tail vein puncture into a VACUPLAST tube of 500 µL capacity, containing serum separator and clot activation factors for biochemical study, as delineated elsewhere with certain adjustments.[23,24] Blood was collected from various subgroups, including Healthy, *W. somnifera* (Ws) treated, *T. cordifolia* (Tc) treated, Tc-Ws treated *L. donovani* contaminated, and AmB-treated *L. donovani*- contaminated mice, and allowed to clot for 30 minutes. Blood aliquots were subjected to centrifugation at 2500 rpm for 5 minutes, and then biochemical parameters were performed for assessment of the blood parameters using dry chemistry methodology.

### Nitric Oxide

Splenocytes were isolated from experimental VL-infected BALB/c mice and processed to obtain a single-cell suspension. The cell density was adjusted to $1 \times 10^6$ cells/mL and seeded into 12-well culture plates. These plates were then incubated in a $CO_2$ incubator for 48 hours. Following incubation, the concentration of nitric oxide (NO) in the culture supernatant was measured using a Griess reagent-based nitric oxide assay kit (Thermo-Fisher™), following the manufacturer's protocol.[25] The NO levels were determined based on a standard curve generated from absorbance readings at 540 nm using a spectrophotometer.

### Reactive Oxygen Species (ROS) Estimation

Intracellular ROS measurements were performed inside the PECs and spleen cells of experimental VL mice. This measurement was performed using 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) (Sigma-Aldrich), as previously described with minor modifications.[15] For this experiment, cells were plated in cell culture plates and administered with 0.4 mM, H2DCFDA Final focus for 15 minutes in the absence of light. Following the incubation period, cells were rinsed with PBS and subsequently lysed using a lysis solution composed of 1% Triton X-100 and 1% SDS in 10 mM Tris. Finally, fluorescence intensity was measured using a spectrofluorometer at 530 nm, with excitation and emission wavelengths set at 488 and 530 nm, respectively.

### Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 software, with results expressed as the mean ± standard error of the mean (SEM). Significance was evaluated using Student's t-test and one-way analysis of variance (ANOVA), followed by Tukey's post hoc multiple comparison test. All experiments were carried out in triplicate, and a *p-value* less than 0.05 was considered statistically significant.

## Results

### Antileishmanial Activity

The findings showed that herbal drugs dissolved in ethanol have a higher antileishmanial activity post 2 weeks of treatment. We analyzed that the no. of LD bodies/1000 cells decreases in a manner based on dosage. The graph shows the no. of LD bodies/1000 cells percentage *versus* different treatment groups of diseased BALB/c mice. The IC50 and IC90 values of *W. somnifera* on amastigotes were observed to be 40 and 80 µg/mL, respectively, while the IC50 and IC90 values of *T. cordifolia* on amastigotes were observed to be 50 and 100 µg/mL, respectively. Both of these drugs were also used in combination at the IC90 value for assessing antileishmanial activity. This combination treatment was performed at a single dose and double dose and concluded that combination treatments of double dose were much more potent than AmB and AmBisome (Fig.1).

### Herbal Extracts Modulate Th1/Th2 Cytokines Produced by T Lymphocytes

In order to efficiently generate immune responses against intracellular infections such as *Leishmania* parasites, cytokines play a crucial role in triggering Th1/Th2 dichotomy. Certain cytokines trigger Th1 cells, which boost macrophage phagocytosis and the removal

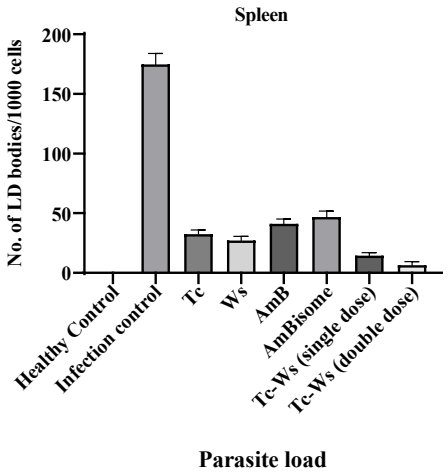


**Fig. 1:** Evaluation of the parasite load in various BALB/c animal cohorts. The experimental spleen tissue's parasite burden was evaluated in *V. leishmaniasis* mice under various treatment circumstances and measured as LD bodies per 1000 cells, represented in a bar plot.

of infected cells, as well as cell-mediated immunity, thereby mediating the death of intracellular pathogens. Conversely, Th2 cells produce a varied reaction when[26] directed by different cytokines. They are essential for allergic reactions and helminth parasite defense, and they mainly target extracellular threats. It is essential to comprehend how cytokines and T cell phenotypes interact in order to customize efficient immunological reactions. Consequently, we compared the levels of Th1 and Th2 cytokines in the splenocytes of BALB/c mice in various treatment groups. We found an elevated concentration of Th2 cytokines, such as IL-10, in infection control, whereas Th1 cytokines, including IFN-γ and IL-12, were considerably low in healthy control and infection control. In treatment groups of BALB/c mice, Th1 cytokines, including IFN-γ and IL-12, were markedly increased. Particularly, Th1 cytokines, including IFN-γ, were discovered 2.7 and 3-fold higher in Tc-Ws single dose and double dose, respectively, when compared to infection control. IL-12 cytokines were also 1.5 and 1.8 fold higher in Tc-Ws single dose and double dose, respectively, when compared to infection control (Fig.2).

## Nitric Oxide Estimation

Nitric oxide (NO) is the pivotal effector element that facilitates the eradication of the Leishmania parasite and is mainly released by infected macrophages. The role of NO in *Leishmania* parasite killing and its secretion level in cell supernatant was monitored in each case after treatment with AmB, AmBisome, Ws, Tc and Tc-Ws (single and double dose). In the case of Tc-Ws (double dose) treated BALB/c mice, we found a 2.8-fold increase in the nitric oxide level when compared to infection controls (Fig. 3).

## Reactive Oxygen Species (ROS) Measurement

Results showed that ROS secretion was markedly increased in the treated group Tc-Ws in the *L. donovani* contaminated BALB/c animal (Fig. 4). The ROS levels measured in contaminated BALB/c mice were comparatively low than in other treatment groups. ROS production measured in the combined treatment condition of Tc-Ws at single dose and double dose showed an increased level when compared with the alone treatment. Combination or alone treatment of these herbal drugs against VL was compared with the standard drug AmB and AmBisome.

## Measurement of Biochemical Parameters

Biochemical parameters assessment was done in the VL-infected mice treated with AmB, AmBisome, Tc (IC90),

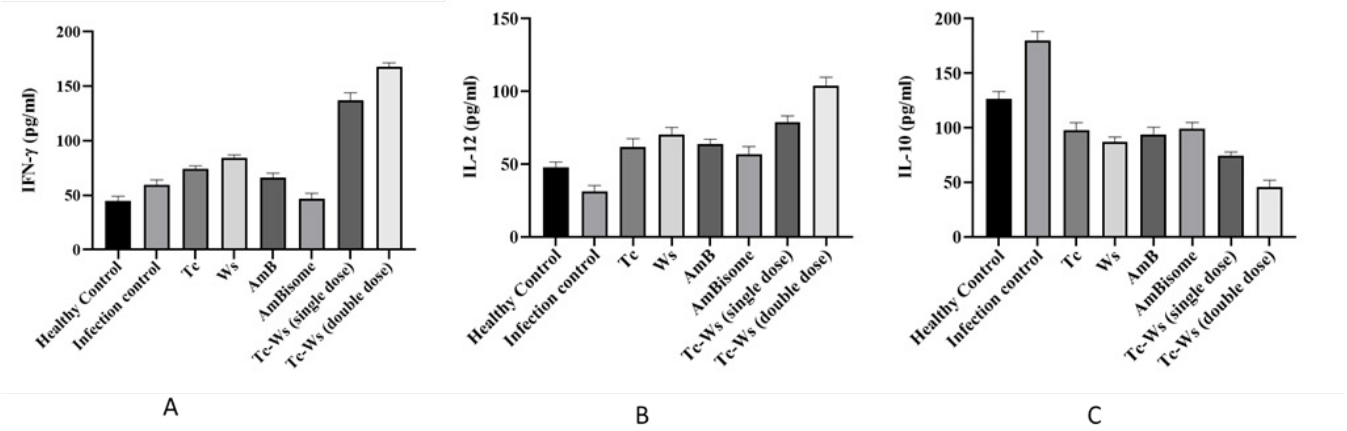


**Fig. 2:** Expression of Th1 cytokines (A & B) and Th2 cytokines (C). Th1 cytokine levels of expression, including as (A) IFN-γ B) IL-12, were up-regulated in different treatment conditions when compared to healthy control and infection control. Whereas Th2 cytokines like (C) IL-10 were significantly up-regulated in the infection control and healthy control when compared to different treatment groups. The cytokines were assessed via the ELISA method in splenocytes extracted from a diverse cohort of empirical BALB/c animals.

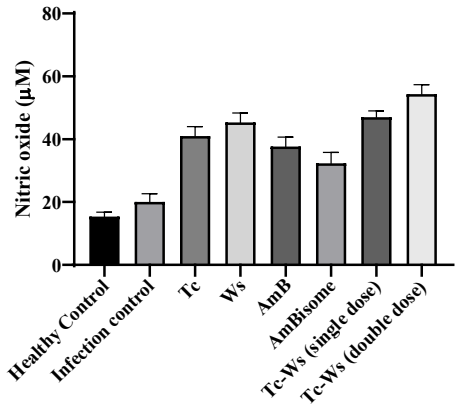


**Fig. 3:** Quantitative assessment of nitric oxide (NO) production (µM) was conducted in the culture supernatant of splenocytes. Splenocytes ($1 \times 10^6$ cells/well) were seeded into a 12-well microtiter chamber slide and subsequently infected with *Leishmania* parasites. The infected splenocytes were treated with AmB, AmBisome, Tc, Ws, and Tc-Ws (single dose and double dose), incubated in the CO2 incubators for 48 hours at 37°C.

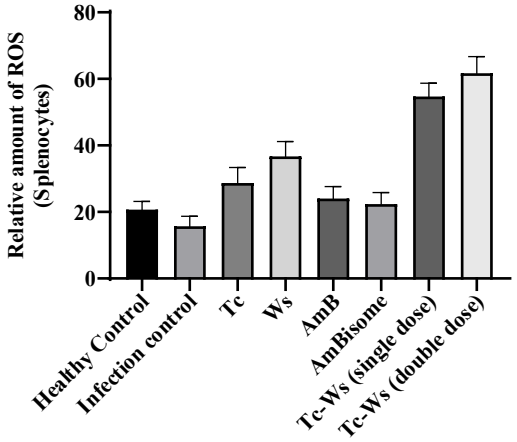


**Fig. 4:** The bar graph shows increased ROS production in splenocytes upon treatment with AmB, AmBisome, and Tc-ws (single dose and double dose). Markedly differences results were observed between healthy controls, infection controls, and treated BALB/c mice. Cells treated with AmB and AmBisome were considered as +ve controls. Fluorescent dye 2, 7-dichlorodihydrofluorescein diacetate (H2DCFDA) was used to estimate ROS production intracellular in the harvested cell pellet as discussed in the methodology section.

Ws (IC90) and in combination with both these drugs at IC90 value of a single dose and a double dose. Tc and Ws exhibited no significant change in alanine and aspartate transaminase concentrations compared to the control group. A concentration of AmB at 10 mg/kg body weight or above affected the biochemical markers of renal and hepatic health. Both herbal drugs Tc and Ws were used in this experimental study with the aim of providing protection to the liver and kidneys. Elevated concentrations of ALT, AST, AP, creatinine, potassium, and sodium were observed in animals afflicted with L. donovani. Administration of the various doses of Tc and Ws significantly brought the concentrations of these biochemical markers within the normal range when contrasted with the standard reference drug AmB, and no mortality was also reported in infected animals treated with these herbal drugs. The possible mechanism that could be responsible for curing VL by Tc and Ws is its potential properties like antioxidant production, immunomodulatory activity and free-radical generation (Table 1). [27,28]

Group I: Control (Only PBS)
Group II: *Leishmania* infection
Group III: *T. cordifolia* (Tc)
Group IV: *W. somnifera* (Ws)
Group V: Pure AmB
Group VI: AmBisome
Group VII: Tc-Ws (single dose)
Group VIII: Tc-Ws (double dose)

## Discussion

This study sought to assess the therapeutic efficacy of *T. cordifolia* (Tc) and *W. somnifera* (Ws) in the management

**Table 1:** Biochemical parameters of the control group mice, *Leishmania* contaminated mice and differentially treated experimental VL mice.

| ***Variables*** | *ALT (iu/l)* | *AST (iu/l)* | *ALP(u/l)* | *BUN (mg/L)* | *Urea (mg/L)* | *Creatinine(mg/dl)* |
|---|---|---|---|---|---|---|
| Group-I | 91 ± 3 | 21 ± 4.7 | 164 ± 9 | 7.3 ± 1.4 | 11.7 ± 2.6 | 0.72 ± 0.12 |
| Group-II | 94 ± 6 | 26 ± 5.3 | 197 ± 14 | 7.82 ± 2.6 | 12.3 ± 2.2 | 1.1 ± 0.41 |
| Group-III | 95 ± 5 | 34 ± 7.1 | 227 ± 17 | 8.4 ± 3.46 | 12.92 ± 4.3 | 1.12 ± 0.22 |
| Group-IV | 92 ± 6 | 32 ± 6.9 | 209 ± 22 | 7.97 ± 2.23 | 11.4 ± 3.25 | 1.03 ± 0.57 |
| Group-V | 162 ± 14 | 49 ± 8.3 | 347 ± 31 | 12.3 ± 4.3 | 19.72 ± 4.7 | 1.62 ± 0.37 |
| Group-VI | 141 ± 9 | 46 ± 7.4 | 304 ± 19 | 11.92 ± 3.85 | 17.9 ± 3.6 | 1.47 ± 0.65 |
| Group-VII | 104 ± 7 | 37 ± 5.8 | 256 ± 12 | 9.37 ± 1.62 | 14.6 ± 4.1 | 1.09 ± 0.26 |
| Group-VIII | 114 ± 11 | 42 ± 6.4 | 273 ± 16 | 10 ± 3.9 | 14.2 ± 2.7 | 1.17 ± 0.53 |

of *V. leishmaniasis* (VL) with a focus on their ability to modulate the immune response and reduce *Leishmania donovani* infection. This research demonstrates substantial evidence for the antileishmanial efficacy of both herbal medications, individually and in combination, with a strong emphasis on immune modulation and safety.

### Antileishmanial Activity and Dose-dependent Parasite Load Reduction

This study revealed a notable dose-dependent decrease in *L. donovani* parasite load following treatment with Tc and Ws, consistent with previous studies on the antileishmanial potential of herbal remedies.[2,29,30] The combination of Tc and Ws at the IC90 dose resulted in a reduction of ≥95% in amastigotes, which was more effective than standard treatments such as AmB and AmBisome. This outcome suggests that Tc and Ws exhibit potent antileishmanial properties, and the combination therapy may offer a more effective and safer alternative to current therapies, which are often associated with significant side effects.[31]

### Immune Modulation and Enhancement of Th1 Cytokines

A crucial element of this investigation was the regulation of the host's immunological response, specifically aimed at augmenting Th1 responses. Leishmaniasis is characterized by an immunosuppressive Th2-dominant immune response, which hinders effective parasite clearance.[32] Our findings shows that treatment with Tc and Ws significantly increased levels of Th1 cytokines such as IFN-γ and IL-12, while Th2 cytokines like IL-10 remained relatively low. This shift toward a Th1-dominant reacting is essential for eliminating intracellular *Leishmania* parasites, as IFN-γ and IL-12 are crucial for activating macrophages and inducing cell-mediated immunity.[33,34] The Tc-Ws double dose the therapy produced a threefold rise in IFN-γ levels in comparison to infection control, highlighting the potential of these herbal drugs in promoting protective immunity.

### Nitric Oxide (NO) and Reactive Oxygen Species (ROS) Production

The production of NO and ROS is critical for the macrophage-mediated killing of *Leishmania* parasites.[15] This study observed a 2.8-folds increase in NO production in the Tc-Ws (double dose) treatment group relative to the infection control group, which further supports the hypothesis that these herbal drugs enhance macrophage activity. Additionally, ROS production was markedly elevated in the combination treatment group, This aligns with prior research indicating that herbal medicines might induce oxidative stress in pathogens.[35,36] These findings suggest that Tc and Ws may promote the clearance of *Leishmania* parasites through the induction of oxidative stress and NO-mediated parasite killing.

### Biochemical Safety Profile

One of the key advantages of Tc and Ws over conventional therapies like AmB is their favourable safety profile. While AmB and its liposomal formulation AmBisome are effective against VL, they are associated with significant hepatotoxicity and nephrotoxicity.[37,38] In contrast, Tc and Ws did not induce any notable alterations in liver and kidney function markers, indicating that these herbal drugs have minimal toxicity and could offer a safer alternative for VL treatment. This is particularly important for chronic or recurrent infections, where prolonged treatment may be necessary.[2,29,39] The lack of mortality and the normal biochemical parameters in Tc and Ws-treated animals support their potential as non-toxic alternatives to traditional therapies.

### Overall Implications and Future Directions

This study's results offer strong evidence for the potential of Tc and Ws as antileishmanial agents that offer both efficacy and safety. The combination of these herbal drugs demonstrated superior potency compared to standard therapies like AmB and AmBisome. These findings align with prior research that has emphasized the capacity of herbal medications to regulate the immunological response and diminish parasite load in VL.[2,16,29] However, further clinical trials are necessary to confirm the safety and efficacy of Tc and Ws in humans. Additionally, the molecular mechanisms underlying the immunomodulatory and antiparasitic effects of these herbal drugs warrant further investigation.

## Conclusion

In conclusion, *T. cordifolia* and *W. somnifera* are promising antileishmanial agents that offer significant immune modulation, enhanced parasite clearance, and a favorable safety profile. The combination of these herbal drugs, particularly at the IC90 dose, demonstrated superior antileishmanial activity compared to standard drugs such as AmB and AmBisome, with minimal toxicity. Given the increasing concern over the side effects and high costs of current VL treatments, Tc and Ws provide a cost-effective and safer alternative for *V. leishmaniasis* treatment. Further studies, including clinical trials, are necessary to validate these findings and explore their potential for widespread use in the treatment of VL.

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## References

1. McCall LI, Zhang WW, Matlashewski G. Determinants for the Development of *Visceral leishmaniasis* Disease. Chitnis CE, editor.

PLoS Pathog. 2013 Jan 3;9(1):e1003053. Available from: https://dx.plos.org/10.1371/journal.ppat.1003053
2. Kaur S, Chauhan K, Sachdeva H. Protection against experimental *Visceral leishmaniasis* by immunostimulation with herbal drugs derived from *Withania somnifer*a and Asparagus racemosus. J Med Microbiol. 2014;63:1328–38. Available from: https://doi.org/10.1099/jmm.0.072694-0
3. Bhunia GS, Kesari S, Chatterjee N, Kumar V, Das P. The Burden of *Visceral leishmaniasis* in India: Challenges in Using Remote Sensing and GIS to Understand and Control. ISRN Infect Dis. 2013 Nov 4;2013:1–14. Available from: https://doi.org/10.5402/2013/675846
4. Torres-Guerrero E, Quintanilla-Cedillo MR, Ruiz-Esmenjaud J, Arenas R. Leishmaniasis: a review. F1000Res. 2017 May 26; 6:750. Available from: /pmc/articles/PMC5464238
5. Taslimi Y, Zahedifard F, Rafati S. Leishmaniasis and various immunotherapeutic approaches. Parasitology. 2018 Apr 1;145(4):497–507. Available from: https://pubmed.ncbi.nlm.nih.gov/27974063
6. Andargie TE, Diro Ejara E. Pro- and Anti-inflammatory Cytokines in *Visceral leishmaniasis*. J Cell Sci Ther. 2016;06(02). Available from: https://www.omicsonline.org/open-access/pro-and-antiinflammatory-cytokines-in-visceral-leishmaniasis-2157-7013-1000206.php?aid=51612
7. Utsugi M, Dobashi K, Ishizuka T, Endou K, Hamuro J, Murata Y, et al. c-Jun N-Terminal Kinase Negatively Regulates Lipopolysaccharide-Induced IL-12 Production in Human Macrophages: Role of Mitogen-Activated Protein Kinase in Glutathione Redox Regulation of IL-12 Production. The Journal of Immunology. 2003 Jul 15;171(2):628–35. Available from: https://doi.org/10.4049/jimmunol.171.2.628
8. Salih MAM, Fakiola M, Abdelraheem MH, Younis BM, Musa AM, ElHassan AM, et al. Insights into the possible role of IFNG and IFNGR1 in Kala-azar and Post Kala-azar Dermal Leishmaniasis in Sudanese patients. BMC Infect Dis. 2014 Dec 3;14(1). Available from: /pmc/articles/PMC4265480/
9. Murray HW. Targeting IL-27 and/or IL-10 in Experimental Murine *Visceral leishmaniasis*. Am J Trop Med Hyg. 2020 Nov 4;103(5):1938–41. Available from: https://www.ajtmh.org/view/journals/tpmd/103/5/article-p1938.xml
10. Stevens TL, Bossie A, Sanders VM, Fernandez-Botran R, Coffman RL, Mosmann TR, et al. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature. 1988;334(6179):255–8. Available from: https://pubmed.ncbi.nlm.nih.gov/2456466/
11. Shadab M, Jha B, Asad M, Deepthi M, Kamran M, Ali N. Apoptosis-like cell death in Leishmania donovani treated with KalsomeTM10, a new liposomal amphotericin B. PLoS One. 2017 Feb 1;12(2). Available from: 10.1371/journal.pone.0171306
12. Laniado-Laborín R, Cabrales-Vargas MN. Amphotericin B: side effects and toxicity. Rev Iberoam Micol. 2009 Dec 31;26(4):223–7. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1130140609000291
13. Sundar S, Chakravarty J. Liposomal amphotericin B and leishmaniasis: dose and response. J Glob Infect Dis. 2010 May;2(2):159–66. Available from: http://www.jgid.org/text.asp?2010/2/2/159/62886
14. Kumar P, Kumar P, Singh N, Khajuria S, Patel R, Rajana VK, et al. Limitations of current chemotherapy and future of nanoformulation-based AmB delivery for *Visceral leishmaniasis*-An updated review. Front Bioeng Biotechnol. 2022 Dec 14;10. Available from: https://pubmed.ncbi.nlm.nih.gov/36588956/
15. Jamal F, Altaf I, Ahmed G, Asad S, Ahmad H, Zia Q, et al. Amphotericin B Nano-Assemblies Circumvent Intrinsic Toxicity and Ensure Superior Protection in Experimental *Visceral leishmaniasis* with Feeble Toxic Manifestation. Vaccines (Basel). 2023 Jan 1;11(1). Available from: 10.3390/vaccines11010100
16. Kaur R, Kaur S. Evaluation of in vitro and in vivo antileishmanial potential of bergenin rich Bergenia ligulata (Wall.) Engl. root extract against *Visceral leishmaniasis* in inbred BALB/c mice through immunomodulation. J Tradit Complement Med. 2018;8(1):251–60. Available from: http://dx.doi.org/10.1016/j.jtcme.2017.06.006
17. Agarwal R, Diwanay S, Patki P, Patwardhan B. Studies on immunomodulatory activity of *Withania somnifer*a (Ashwagandha) extracts in experimental immune inflammation. J Ethnopharmacol. 1999;67(1):27–35. Available from: doi: 10.1016/s0378-8741(99)00065-3
18. Singh N, Kumar A, Gupta P, Chand K, Samant M, Maurya R, et al. Evaluation of antileishmanial potential of Tinospora sinensis against experimental *Visceral leishmaniasis*. Parasitol Res. 2008;102(3). Available from: doi: 10.1007/s00436-007-0822-2
19. Kushwaha S, Roy S, Maity R, Mallick A, Soni VK, Singh PK, et al. Chemotypical variations in *Withania somnifer*a lead to differentially modulated immune response in BALB/c mice. Vaccine. 2012;30(6):1083–93. Available from: http://dx.doi.org/10.1016/j.vaccine.2011.12.031
20. Ahmed G, Jamal F, Tiwari RK, Singh V, Rai SN, Chaturvedi SK, et al. Arsenic exposure to mouse *Visceral leishmaniasis* model through their drinking water linked to the disease exacerbation via modulation in host protective immunity: a preclinical study. Sci Rep. 2023 Dec 1;13(1). Available from: 10.1038/s41598-023-48642-z
21. Ahmed G, Thakur AK, Pushpanjali, Snehil, Chaturvedi SK, Shivam P, et al. Modulation of the immune response and infection pattern to Leishmania donovani in *Visceral leishmaniasis* due to arsenic exposure: An in vitro study. Afrin F, editor. PLoS One. 2019 Feb 5;14(2):e0210737. Available from: https://dx.plos.org/10.1371/journal.pone.0210737
22. Späth GF, Beverley SM. A lipophosphoglycan-independent method for isolation of infective Leishmania metacyclic promastigotes by density gradient centrifugation. Exp Parasitol. 2001 Oct;99(2):97–103. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0014489401946569
23. Cambridge EL, Mcintyre Z, Clare S, Arends MJ, Goulding D, Isherwood C, et al. The AMP-activated protein kinase beta 1 subunit modulates erythrocyte integrity. Exp Hematol. Available from: doi: 10.1016/j.exphem.2016.09.006
24. Silva-Santana G, Bax JC, Fernandes DCS, Bacellar DTL, Hooper C, Dias AASO, et al. Clinical hematological and biochemical parameters in Swiss, BALB/c, C57BL/6 and B6D2F1 Mus musculus . Animal Model Exp Med. 2020;3(4):304–15. Available from: doi: 10.1002/ame2.12139
25. Pushpanjali, Thakur AK, Purkait B, Jamal F, Singh MK, Ahmed G, et al. Direct evidence for role of anti-saliva antibodies against salivary gland homogenate of P. argentipes in modulation of protective Th1-immune response against Leishmania donovani. Cytokine. 2016 Oct;86:79–85. Available from: http://dx.doi.org/10.1016/j.cyto.2016.07.017
26. Ranneh Y, Ali F, Akim AM, Hamid HA, Khazaai H, Fadel A. Crosstalk between reactive oxygen species and pro-inflammatory markers in developing various chronic diseases: a review. Appl Biol Chem. 2017 Jun 1;60(3):327–38. Available from: doi: https://doi.org/10.1007/s13765-017-0285-9
27. Gaurav H, Yadav D, Maurya A, Yadav H, Yadav R, Shukla AC, Sharma M, Gupta VK, Palazon J. Biodiversity, Biochemical Profiling, and Pharmaco-Commercial Applications of *Withania somnifera*: A Review. Molecules. 2023 Jan 26;28(3):1208. Available from: doi: 10.3390/molecules28031208. PMID: 36770874; PMCID: PMC9921868.
28. Sharma U, Bala M, Kumar N, Singh B, Munshi RK, Bhalerao S. Immunomodulatory active compounds from *Tinospora cordifolia*. J Ethnopharmacol. 2012;141(3). Available from: doi: 10.1016/j.jep.2012.03.027
29. Sachdeva H, Sehgal R, Kaur S. *Tinospora cordifolia* as a protective and immunomodulatory agent in combination with cisplatin against murine *Visceral leishmaniasis*. Exp Parasitol. 2014 Feb; Gaurav H, Yadav D, Maurya A, Yadav H, Yadav R, Shukla AC, et al. Biodiversity, Biochemical Profiling, and Pharmaco-Commercial Applications of *Withania somnifer*a: A 137:53–65. Available from: http://dx.doi.org/10.1016/j.exppara.2013.12.006
30. Dayakar A, Chandrasekaran S, Veronica J, Sundar S, Maurya R. In vitro and in vivo evaluation of anti-leishmanial and

immunomodulatory activity of Neem leaf extract in Leishmania donovani infection. Exp Parasitol. 2015;153:45–54. Available from: http://dx.doi.org/10.1016/j.exppara.2015.02.011
31. Mann S, Frasca K, Scherrer S, Henao-Martínez AF, Newman S, Ramanan P, et al. A Review of Leishmaniasis: Current Knowledge and Future Directions. Vol. 8, Current Tropical Medicine Reports. 2021. Available from: doi: 10.1007/s40475-021-00232-7
32. Ghosh A, Zhang WW, Matlashewski G. Immunization with A2 protein results in a mixed Th1/Th2 and a humoral response which protects mice against Leishmania donovani infections. Vaccine. 2001;20(1–2). Available from: doi: 10.1016/s0264-410x(01)00322-x
33. Afrin F, Chouhan G, Islamuddin M, Want MY, Ozbak HA, Hemeg HA. Cinnamomum cassia exhibits antileishmanial activity against Leishmania donovani infection in vitro and in vivo. Oliveira F, editor. PLoS Negl Trop Dis. 2019 May 9;13(5):e0007227. Available from: https://dx.plos.org/10.1371/journal.pntd.0007227
34. Cláudio Nascimento Da Silva L, Carolina Accioly Brelaz De M, Afrin afrin F, Chouhan G, Islamuddin M, Want MY, et al. Leishmanicidal Activity of Piper nigrum Bioactive Fractions is Interceded via Apoptosis In Vitro and Substantiated by Th1 Immunostimulatory Potential In Vivo. 2015; Available from: www.frontiersin.org
35. Arunachalam K, Yang X, San TT. *Tinospora cordifolia* (Willd.) Miers: Protection mechanisms and strategies against oxidative stress-related diseases. Vol. 283, Journal of Ethnopharmacology. 2022. Available from: doi: 10.1016/j.jep.2021.114540
36. Melcon-Fernandez E, Galli G, García-Estrada C, Balaña-Fouce R, Reguera RM, Pérez-Pertejo Y. Miltefosine and Nifuratel Combination: A Promising Therapy for the Treatment of Leishmania donovani *Visceral leishmaniasis*. Int J Mol Sci. 2023;24(2). Available from: doi: 10.3390/ijms24021635
37. He J, Chipot C, Shao X, Cai W. Cyclodextrin-Mediated Recruitment and Delivery of Amphotericin B. The Journal of Physical Chemistry C. 2013 Jun 6;117(22):11750–6. Available from: https://pubs.acs.org/doi/10.1021/jp3128324
38. Roatt BM, de Oliveira Cardoso JM, De Brito RCF, Coura-Vital W, de Oliveira Aguiar-Soares RD, Reis AB. Recent advances and new strategies on leishmaniasis treatment. Vol. 104, Applied Microbiology and Biotechnology. 2020. Available from: doi: 10.1007/s00253-020-10856-w
39. Ponte-Sucre A, Gamarro F, Dujardin JC, Barrett MP, López-Vélez R, García-Hernández R, et al. Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. Maes L, editor. PLoS Negl Trop Dis. 2017 Dec 14;11(12):e0006052. Available from: https://dx.plos.org/10.1371/journal.pntd.0006052

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